Identification of the principal serological immunodeterminants of African swine fever virus by screening a virus cDNA library with antibody

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Protective immunity to African swine fever virus (ASFV) may involve a combination of both serological and cellular mechanisms. This work is focused on the identification of the possible relevant serological immunodeterminants of immunity. Thus, 14 serological immunodeterminants of ASFV have been characterized by exhaustive screening of a representative lambda phage cDNA expression library of the tissue culture-adapted Ba71V strain of ASFV. The library was constructed using RNA extracted from Vero cells infected for 3, 6, 9 and 12 h. A total of 150 clones was selected arbitrarily by antibody screening of the library with a polyclonal antiserum from a domestic pig surviving infection with the virulent Malta isolate of ASFV. Sequencing of these clones permitted identification of 14 independent viral proteins that stimulated an antibody response. These included six proteins encoded by previously unassigned open reading frames (ORFs) (B602L, C44L, CP312R, E184L, K145R and K205R) as well as some of the more well-studied structural (A104R, p10, p32, p54 and p73) and non-structural proteins (RNA reductase, DNA ligase and thymidine kinase). Immunogenicity of these proteins was confirmed by demonstrating the corresponding antibodies in sera from pigs infected either with the Malta isolate or with the OURT88/3–OURT88/1 isolate combination. Furthermore, the majority of these ORFs were also recognized by immune antiserum from the natural host, the bush pig, following secondary challenge with the virulent Malawi (SINT90/1) isolate of ASFV. Thus, it is possible that some of these determinants may be important in protection against virus infection.

Introduction

African swine fever virus (ASFV) is a large DNA virus that shares several structural features with iridoviruses but has a genomic organization similar to poxviruses. The genome varies between 170 and 190 kb in size, depending on the isolate. Sequencing of the complete genome of the Ba71V isolate of ASFV suggests that the virus may encode 151 major open reading frames (ORFs) of 60 or more amino acids and as many as 160 additional minor ORFs (Yañez et al., 1995). Both DNA chains are alternatively used as the coding strand (Dixon et al., 1994; Yan ez et al., 1995). Up to 40 of the polypeptides synthesized by ASFV in pig macrophages may be incorporated into the virus particle (Alcaraz et al., 1992; Carrascosa et al., 1985; Esteves et al., 1986).

ASFV was first observed when it emerged causing a typically lethal haemorrhagic disease of domestic pigs (Sus scrofa) (Montgomery, 1921). This is in complete contrast to the absence of disease symptoms in the natural hosts of ASFV in sub-Saharan Africa, the bush pig (Potamochoerus porcus) and the warthog (Phacochoerus aethiopicus), where the virus persists. Later, ASFV was shown to infect soft ticks (Ornithodoros moubata and O. erraticus), which are ectoparasites of warthogs and, on occasion, domestic pigs, thereby creating a cycle of infection that maintains the virus in Africa and elsewhere.

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(Costa, 1990; Viñuela, 1985; Wilkinson, 1989). Since its initial isolation, a number of less virulent forms have evolved and a range of isolates, causing from quickly lethal to very attenuated and chronic infections in domestic pigs, has been described (Mebus, 1988). The sequences of the virulent Malawi and non-pathogenic Ba71V isolates of the virus share 92% identity and the majority of proteins, including structural subunits encoded by different isolates, are either identical or highly similar (Dixon et al., 1994; Yañez et al., 1995).

Swine infected with ASFV develop anti-viral antibodies that can be demonstrated from 7 days post-infection (p.i.) by a wide variety of tests (Cowan, 1961; Crowther et al., 1979; Hamdy et al., 1981; Malmquist, 1963; Pan et al., 1972, 1974, 1982; Parker & Plowright, 1968). Pigs that recover are usually resistant to challenge with homologous, but not heterologous, virus isolates (Ruiz-Gonzalvo et al., 1984; Schlafer et al., 1984). This isolate-specific immunity is not understood but it is unlikely to result from variation in antibody responses against different isolates, as both virulent and non-pathogenic isolates have a shared core of serological immunodeterminants.

Although there is no formal evidence for classical anti-virus neutralizing antibodies, antibody responses can clearly contribute to protection. Passive administration of serum or colostrum from recovered, convalescent animals can delay the onset of clinical symptoms in infected pigs, reduce viraemia and enhance survival (Coggins, 1968, 1974; Malmquist, 1963; Parker & Plowright, 1968). Pigs that recover are usually resistant to challenge with homologous, but not heterologous, virus isolates (Ruiz-Gonzalvo et al., 1986; Schlafer et al., 1984). This isolate-specific immunity is not understood but it is unlikely to result from variation in antibody responses against different isolates, as both virulent and non-pathogenic isolates have a shared core of serological immunodeterminants.

In terms of possible cellular mechanisms of resistance to infection, there is some evidence for the participation of cytotoxic T cells (Martins et al., 1993; Alonso et al., 1997; Leitão et al., 1998; Jenson et al., 2000).

The weight of evidence, therefore, indicates an important (but not necessarily exclusive) role for antibodies in the protective antibody response of pigs to ASFV but the exact mechanism and range of critical serological virus immunodeterminants have yet to be defined. Thus, in order to systematically identify potentially protective serological immunodeterminants, we have screened a representative lambda phage cDNA expression library of the ASFV Ba71V isolate with polyclonal immune antisera from susceptible domestic pigs and resistant bush pigs, both recovered from infection with virulent isolates of the virus. In this way, we have identified 14 viral ORFs that encode serological epitopes of the virus. These included four structural proteins, three non-structural proteins and six viral subunits with unassigned functions. Several of these proteins elicited antibody responses comparable to the important structural immunodeterminants p32 and p73 in domestic pigs protected from infection with the virulent OURT88/1 isolate by previous infection with the related non-pathogenic OURT88/3 isolate of the virus. The fact that the majority of these proteins were also recognized by immune antisera from the natural host, the bush pig, suggests that some of these determinants may play a role in protection against virus infection.

Methods

Antiserum used for expression screening of the ASFV cDNA library. The ASFV cDNA library was screened with a polyclonal antiserum (MI92) against the virulent Malta isolate from an outbred pig that had survived infection with the virus.

Antiserum from domestic pigs infected with the Malta isolate of ASFV. An outbred pig (Landrace–Large White cross) was infected with $10^4$ HAD$_{50}$ (50% haemadsorbing doses) of the virulent Malta isolate of ASFV. Blood for antiserum preparation for ELISA was taken on day 0 and 28 days after infection.

Longitudinal antiserum was collected from a domestic pig infected with the non-pathogenic OURT88/3 isolate of ASFV and subsequently challenged with the virulent OURT88/1 isolate.

An outbred pig was infected with $10^4$ HAD$_{50}$ of the avirulent Portuguese isolate OURT88/3. Pigs infected with OURT88/3 are subsequently immune to the related highly virulent OURT88/1 isolate. The pig was challenged on day 28 with OURT88/1. ELISAs were performed with serial dilutions of antiserum prepared from blood taken 6 days after challenge with OURT88/1.

Bush pig antiserum. Bush pig antiserum was obtained from Dr Euan Anderson, Veterinary Research Laboratory, Harare, Zimbabwe. A 6-month-old bush pig, captured from an ASFV-free area in Zimbabwe, was intramuscularly inoculated with $10^3$ HAD$_{50}$ of the Malawi (SINT90/1) isolate of ASFV (Haresnape et al., 1988). Blood for antiserum preparation was taken on day 0, before primary infection with the virus, and 307 days after a secondary challenge with the SINT90/1 isolate of the virus on day 217.

Generation of an ASFV lambda cDNA expression library

Infection of Vero cells with the Ba71V isolate of ASFV and isolation of polyadenylated RNA. VERO cells ($2 \times 10^7$) at 80% confluence were infected (m.o.i. of 10) with the Portuguese Ba71V isolate for 3, 5, 6, 9 and 12 h. Polyadenylated RNA was isolated from cells at each time-point using the Poly(A) Tract 1000 system (Promega). Samples of 1-25 ng mRNA isolated from each time-point were pooled and the resulting 5 µg was used for cDNA synthesis.
cDNA synthesis and size selection. Synthesis of cDNA, adaptor ligations, enzyme digestions, phosphorylations and size selection were performed according to the manufacturer’s instructions using the Lambda ZAP Library system (Stratagene).

cDNA was pooled into three fractions: high, medium and low, each containing DNA of sizes 2–0, 1–0–2–0 and 0–4–10 kb, respectively. Each fraction was used to generate a cDNA library in the Lambda-ZAP Phage Expression system.

Each of the three cDNA fractions was ligated into the phage vector, packaged and titred, as described in the Stratagene manual.

Purification of plaques, plaque excision and sequencing

Expression screening. IPTG induction of phage protein expression and plaque lifts were performed as described previously (see Stratagene manual). After protein induction, membranes (Hybond C) (Amersham–Pharmacia) were removed from the plates, washed with PBS and blocked with 5% Marvel (w/v) in PBS (blocking buffer). Subsequently membranes were incubated at 4 °C overnight with porcine anti-ASFV Malta serum M102 (1:2000 in blocking buffer) and developed with DAB (Sigma) after incubation for 1 h at room temperature with HRP-conjugated rabbit anti-pig immunoglobulins (Dako) at a 1:1000 dilution. Positive plaques were then picked and purified and single clone excisions were performed as outlined in the Stratagene manual.

Sequencing and sequence alignment. Miniprep DNA for sequencing was prepared from excised phagemids using the alkaline lysis method (Sambrook et al., 1989). Sequencing was performed with 5 μg DNA template using the Cy5 Autoread Sequencing kit (Amersham–Pharmacia) and an ALF Automated DNA Sequencing machine (Amersham–Pharmacia). Sequence analyses and manipulation were performed using the EasyGCG8 program and the NCBI database (http://www.ncbi.nlm.nih.gov).

PCR primer design, PCR and subcloning. PCR primers for the viral ORFs A151R, C44L, E184L, K76R and K145R were designed with reference to the database, incorporating appropriate restriction sites for subcloning in pGEX 4T-1 (Amersham–Pharmacia). In the case of ORFs C44L and E184L, a combination of a 3’ T primer from the parental pBK-CMV vector and a 5’ N-terminal primer specific for these ORFs were used. All PCRs were performed with high-fidelity Pfu polymerase (Promega). PCR products were cloned into the pGEX 4T-1 prokaryotic expression vector and the resulting constructs were used to transform competent Escherichia coli, strain DH5-α (Gibco), for expression and analysis of reactivity with immune antisera.

Purification of glutathione S-transferase (GST)-fusion proteins. Overnight cultures of E. coli transformed with ASFV cDNA recombinants in pGEX grown in select medium were diluted 1:50 in 100 ml fresh LB medium containing ampicillin and grown for 3–5 h to the mid-exponential phase. Expression was induced for 2 h with 0.1 mM IPTG. Cells were harvested by centrifuging at 2500 g for 10 min, resuspended in ice-cold PBS and lysed with B-Per Extraction agent (Pierce). ORFs p54/E183L, p32/CP204L, histone-like protein/A104R, thymidine kinase/K196R, K205R and C44L were purified as soluble proteins on glutathione–Sepharose 4B columns (Amersham–Pharmacia), as described by the manufacturer. The insoluble proteins p73/B646L, p10/K78R, DNA ligase/NP419L, small subunit of ribonucleotide reductase/F334L, B602L and CP312R were solubilized from inclusion bodies with 6 M guanidine hydrochloride prior to resolving by SDS–PAGE, as described previously (Katrak et al., 1992).

ELISA. Nunc ELISA plates were coated with purified ASFV fusion proteins or purified Schistosoma japonicum GST (Sigma) (2 μg/ml at 50 μl per well) in coating buffer (0.05 M Na₂CO₃/NaHCO₃ pH 9.6) and incubated overnight at 4 °C. Plates were washed and blocked, as described previously (Cedillo-Barron et al., 2001), and incubated for 1 h at 37 °C with serial doubling dilutions of test sera in PBS. Plates were developed, as described previously (Cedillo-Barron et al., 2001), and absorbances were read at 492 nm on a Titretek Multiskan spectrophotometer.

Analytical SDS–PAGE and Western blots. Proteins were separated by denaturing SDS–PAGE using 10 or 15% (v/v) acrylamide–bisacrylamide resolving gels and a 4% (v/v) acrylamide–bisacrylamide stacking gel. Biorad molecular mass markers were used as standards.

Western blots were performed using Immobilon membrane (Millipore) and a semidry Western blotter (Sartoblot II), as described previously (Cedillo-Barron et al., 2001). After blocking, membranes were incubated for 1 h at 37 °C with immune antisera (diluted in blocking buffer) from pigs that had recovered from virus infection, washed with PBS–Tween, incubated with HRP-conjugated rabbit anti-pig immunoglobulins (1:1000 in blocking buffer) for 1 h at 37 °C and developed with DAB.

Results

Representation of virus cDNAs in the BA71V virus cDNA expression library

Early ASFV RNA synthesis begins 2 h p.i. (Yañez et al., 1995), after which there is a stage of intermediate viral RNA synthesis peaking 6–8 h p.i. (Rodríguez et al., 1996), with late viral RNA synthesis and formation of virus factories starting from 8 to 10 h p.i. (Yañez et al., 1995; Brookes et al., 1996). Thus, the Ba71V virus isolate cDNA expression library was constructed using a mixture of polyadenylated RNA isolated from Vero cells that had been infected with the virus for 3, 6 and 12 h.

Three fractions of cDNA in the size ranges of 0–2–10, 1–0–2–0 and > 200 kb were used separately to generate three corresponding cDNA libraries with 176120, 199060 and 185738 recombinants, respectively. These three fractions were chosen in order to include a representative range of viral ORFs, taking into account that ASFV ORFs range from 182 bp to 4 kb in size, with an average size of 987 bp.

Between 6 and 10% of plaques from each of the unamplified phage libraries were shown to contain virus inserts by colony filter hybridization using Malawi isolate viral DNA as a probe (Table 1).

Between 0.6 and 1% of plaques from each of the three unamplified phage libraries expressed serological virus epitopes identified with a polyclonal antiserum (M192) from a domestic pig that had recovered from infection with the virulent Malta isolate of ASFV (Table 1). This antiserum was chosen for expression screening because of its high titre of anti-ASFV antibodies.

As a further assessment, DNA isolated from the phage library by PEG precipitation was screened by PCR with specific primers for the viral ORFs. Full-length PCR products were
Sequencing of purified viral cDNAs isolated from the library

A total of 50 plaques was arbitrarily purified from each of the three different cDNA libraries (low, medium and high) and miniprep DNA from excised phagemids was prepared for sequencing. Clones were identified by sequencing from the N- and C-terminal ends of the cDNA and viral ORFs were assigned with reference to the database. The contiguity of intervening sequence was confirmed by restriction mapping, comparing the pattern of restriction fragments observed with that predicted from the published sequence of the Ba71V isolate (results not shown).

Following this analysis, 18 unique viral cDNAs were defined. Six were fragments of other larger cDNAs that were independently isolated. The remaining 12 cDNAs incorporated sequences from 18 viral ORFs, with the size of these cDNAs ranging from 0.35 to 2.9 kb (Table 2).

Five of the isolated cDNAs were polycistronic. These clones were as follows: clone 1, a 2.9 kb cDNA encoding ORFs A104R, A118R, A151R and 694 bp of A276R; clone 2, a 2.3 kb transcript incorporating ORF B002L and 456 bp of the adjacent ORF B354L; clone 3, a 1.05 kb cDNA encoding 281 bp of ORF E183L and the adjacent ORF E184L; clone 9, a 1.7 kb cDNA encoding ORFs K205R, K78R, K196R and K145R; and clone 10, a 1.1 kb cDNA encoding the viral ORFs thymidine kinase/K196R and K145R (Fig. 1 and Table 2).

Antigenicity of viral subunits coded by monocistronic cDNAs

In order to confirm the immunogenicity of the viral subunits selected by the polyclonal antiserum used to screen the library, IPTG-induced bacterial lysates from excised phagemids were analysed by Western blot analysis using polyclonal porcine anti-ASFV serum.

After preabsorption against bacterial lysate, polyclonal antisera from recovered domestic pigs consistently showed no cross reaction with IPTG-induced bacterial lysates from the XLOLR host strain used for phagemid excision and E. coli strain DH5α used for subcloning (Fig. 2B, C, lanes 2 and 4). In addition, preabsorbed antisera failed to react with lysates from several ASFV cDNA constructs generated by restriction digestion (Fig. 3). Fusion proteins with the predicted molecular masses from pBK-CMV were identified for each of the cDNAs encoding B002L (~85 kDa), p32/CP204L (~33 kDa), CP312R (~37 kDa), K205R (~24 kDa) and DNA ligase/ NP419L (~42 kDa) (Fig. 2). ORFs small ribonucleotide reductase subunit/F334L and p73/B646L were not clearly recognized by Western blot when production of bacterial proteins was induced by IPTG in the pBK-CMV phagemid vector used to generate the library (results not shown). However, both of these viral subunits were clearly identified by ELISA (see below and Figs 5 and 6) after subcloning of the respective cDNAs (clones 5 and 6) in the pGEX expression system and sensitization of ELISA plates with purified GST fusion proteins. In addition, a 100 kDa GST fusion protein of the viral p73 structural protein was also identified by immune antiserum (results not shown). Antisera from immune domestic pigs and bush pigs clearly recognized both these proteins when protein expression was induced in the purified phage corresponding to these two ORFs (results not shown). The absence of a positive Western blot with the ribonucleotide reductase subunit/F334L may be explained by a requirement for conformationally dependent epitopes.

Clone 12 encoded a minor ORF of 44 amino acids from the virus, which we have tentatively named C44L because of its position in the viral genome (Fig. 4A, C). As this ORF was not detected by Western blot when expressed in the parental plasmid pBK-CMV, the PCR product corresponding to this ORF was cloned from its corresponding isolated cDNA into

<table>
<thead>
<tr>
<th>Size fraction of cDNA (kb)</th>
<th>Quantity of cDNA (ng)</th>
<th>No. of recombinants</th>
<th>No. of plaques recognized by Southern blot Malawi DNA probe (%)</th>
<th>No. of plaques recognized by M192 antisera (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High (&gt; 2.0)</td>
<td>100</td>
<td>185 738</td>
<td>11 430 (6.0)</td>
<td>1 143 (0.6)</td>
</tr>
<tr>
<td>Medium (1.0–2.0)</td>
<td>250</td>
<td>190 006</td>
<td>17 063 (7.5)</td>
<td>1 593 (0.7)</td>
</tr>
<tr>
<td>Low (0.4–1.0)</td>
<td>150</td>
<td>176 120</td>
<td>17 580 (10)</td>
<td>1 760 (1)</td>
</tr>
</tbody>
</table>

Table 1. Summary of the viral cDNA library characteristics

Nylon filter replicas were taken from plates of 10 000 plaques from each of the three cDNA library fractions (low, medium and high) used to generate the library. Filters were either hybridized with a 32P-labelled Malawi DNA probe or developed with antisera from a domestic pig infected with the Malta isolate of ASFV (MI92) after induction of viral protein expression with IPTG. The total number of plaques identified by each of these two respective methods was then calculated for the unamplified libraries.
Table 2. Summary of cloned cDNAs isolated from the Ba71V cDNA library and viral proteins recognised by immune antisera from domestic pigs and bush pigs

cDNAs were purified by expression screening of an ASFV lambda phage cDNA library with antisera from domestic pigs and bush pigs recovered from infection with virulent isolates of ASFV (Malta and Malawi isolates, respectively).

<table>
<thead>
<tr>
<th>Clone</th>
<th>Size of cloned cDNA (kb)*</th>
<th>Sequence alignment with BA71V genome N- to C-terminal (bp)</th>
<th>ORFs within the cDNA (kDa)†</th>
<th>Viral subunits recognized by immune antisera‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.85</td>
<td>30 184–32 991</td>
<td>A104R (11.0)</td>
<td>A104Rβ-gal fusion proteins (14.0)$</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>A118R (13.8)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>A151R (17.6)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>A276R (31.6)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>A240L (27.8)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>B602L (68.0)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>B354L (41.7)</td>
<td></td>
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<tr>
<td>2</td>
<td>2.3</td>
<td>85 479–83 207</td>
<td>p32/CP204L (32.0)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>K205R (23.7)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>K78R (10.0)</td>
<td></td>
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<tr>
<td>3</td>
<td>0.6</td>
<td>108 550–107 915</td>
<td>p73/B464L (72.0)</td>
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<tr>
<td>4</td>
<td>0.8</td>
<td>46 094–46 744</td>
<td>p32/CP204L (32.0)</td>
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</tr>
<tr>
<td>5</td>
<td>2.1</td>
<td>88 716–86 729</td>
<td>p73/B464L (72.0)</td>
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<tr>
<td>6</td>
<td>1.1</td>
<td>39 890–38 872</td>
<td>Ribonucleotide reductase/F334L (39.8)</td>
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<tr>
<td>7</td>
<td>1.1</td>
<td>145 904–144 645</td>
<td>p54/E183L (22.0)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>E184L (22.0)</td>
<td></td>
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<tr>
<td>8</td>
<td>1.1</td>
<td>110 480–111 581</td>
<td>CP312R (35.1)</td>
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</tr>
<tr>
<td>9</td>
<td>1.1</td>
<td>46 423–48 089</td>
<td>K205R (23.7)</td>
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<td>K78R (10.0)</td>
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<td></td>
<td></td>
<td></td>
<td>K196R (22.4)</td>
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<tr>
<td>10</td>
<td>1.1</td>
<td>46 922–48 089</td>
<td>K196R (22.4)</td>
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<td></td>
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<td></td>
<td>K145R (17.2)</td>
<td></td>
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<tr>
<td>11</td>
<td>2.2</td>
<td>117 705–115 773</td>
<td>DNA ligase/NP419L (48.2)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0.4</td>
<td>64 496–64 639</td>
<td>C44L (5.6)</td>
<td></td>
</tr>
</tbody>
</table>

* Refers to the largest DNA fragment identified.
† Where the cDNAs are derived from a polycistronic viral mRNA, the different ORFs contained within the cDNA are listed.
‡ Viral subunits recognised by antisera from infected pigs.
§ ORFs recognised by both antisera from recovered bush pigs and recovered domestic pigs infected with the Malawi and Malta isolates of the virus, respectively, either by ELISA or by Western blot.
∥ ORFs recognised by antisera from domestic pigs infected and challenged with the OURT88 isolates of ASFV.
¶ ORFs recognised by antisera from Malta-infected domestic pigs only.

pGEX. The predicted molecular mass of this protein is approximately 6 kDa and a GST fusion protein of approximately 33 kDa was recognized by immune antisera upon Western blot analysis, verifying that this minor ORF was indeed being recognized (Fig. 4B).

Antigenicity of viral subunits coded by polycistronic cDNAs

The polycistronic sequences presented the problem of how to define which ORF or ORFs were in fact translated into protein recognized by antibodies in the infected pig serum. An additional complication is created by the observation that translation initiation at bases apart from those coded by the initial start codon has been reported for ASFV cDNAs (Cistue et al., 1992; Irusta et al., 1996) and so it was necessary to conclusively demonstrate the identity of the viral subunits recognized by Western blot. Thus, in order to unambiguously define the serological immunodeterminants recognized by immune sera in those cases where the viral cDNA sequence extended beyond the termination signal of a discrete ORF,
successive ORFs within the polycistronic cDNA constructs were removed by restriction digestion and the resulting constructs were then analysed by Western blot. This approach was necessary because more than one protein with molecular masses corresponding to those coded by the ORFs from some of the polycistronic cDNAs was identified by Western blot (Fig. 2 and Table 2). Restriction enzymes were chosen so that, following digestion and generation of the corresponding construct, relevant viral ORFs would be expressed in-frame as a β-galactosidase fusion from the Lac promoter of the pBK-CMV vector. Where this approach was impractical, single viral ORFs, contained within a polycistronic message, were generated by PCR with virus-specific primers using the respective isolated cDNAs as template for subcloning and expression in pGEX.

Thus, clone 1 (A104R, A118R, A151R and A276R) was digested with XmnI/XhoI to remove all of the ORFs downstream of A104R. A 15 kDa β-galactosidase fusion protein of the predicted size of ORF A104R expressed from the resulting construct was positively identified by Western blot with MI92 antiserum (Fig. 3).

An 85 kDa β-galactosidase fusion protein of the predicted molecular mass of ORF B602L was expressed from clone 2 (B602L and B354L) and identified by Western blot (Fig. 2).
Serological immunodeterminants of ASFV

Fig. 2. Characterization of viral proteins expressed from the isolated viral cDNAs identified by immune antisera from ASFV immune pigs. Protein expression from excised phagemids was induced as described in Methods and samples were analysed on 10 or 15% acrylamide–bisacrylamide gels and Western blotted. Membranes were developed with DAB after incubation with appropriate dilutions of immune pig antisera (MI92) and rabbit anti-pig immunoglobulins conjugated to HRP. (A) Analysis of proteins expressed from the isolated viral cDNAs indicated. (B) IPTG-induced bacterial lysates of the host strains of bacteria (E. coli strains XLOLR and DH5-α) were run as negative controls (lanes are indicated). The molecular masses of the proteins identified and the identities of each of the isolated viral cDNAs are indicated. (C) Coomassie blue-stained gel of the control samples in (B).

Clone 10 (K196R and K145R) was digested with XhoI to remove the downstream K145R ORF and the resulting construct obtained after religation and transformation was digested with EcoRI/EcoRV to generate a truncated cDNA encoding ORF thymidine kinase/K196R in-frame with N-terminal β-galactosidase from the pBK-CMV vector. In this way, a 15 kDa β-galactosidase fusion protein of the correct size of the truncated C-terminal domain of thymidine kinase/K196R was positively identified by Western blot with MI92 antisera (Fig. 3).

ORFs A151R, E184L, K78R and K145R were all subcloned as PCR products from clones 1, 7 and 9, respectively, in pGEX. GST fusion proteins of K78R, K145R and E184L of the predicted sizes of 36, 44 and 46 kDa were all positive when probed with porcine anti-ASFV MI92 antiserum by Western blot (Fig. 3). The A151R GST fusion protein was not recognized, although a GST fusion protein of the predicted size of ORF A151R was identified by Coomassie blue staining (Fig. 3).

Fig. 3. Characterization of individual viral subunits from isolated polycistronic cDNAs recognized by immune antisera MI92. Recombinant viral protein expression was induced as described in Methods. Bacterial cell lysates containing induced viral proteins were analysed on 10 or 15% polyacrylamide gels and Western blotted. Membranes were developed with DAB after incubation with appropriate dilutions of immune pig antiserum (MI92) and rabbit anti-pig immunoglobulins conjugated to HRP. The molecular masses of the proteins identified and the identities of each of the isolated cDNAs are indicated. The corresponding Coomassie blue-stained gels of viral ORFs (indicated with an asterisk), subcloned and induced in the pGEX expression system, are also shown in comparison with the 27.5 kDa GST protein expressed in the pGEX vector as a negative control.

Recognition of isolated recombinant viral proteins by antibodies from ASFV-infected domestic pigs and bush pigs

In order to determine which isolated viral subunits may play a key role in protective immunity against the virus, ELISAs were performed with purified recombinant GST fusion proteins. Sensitised ELISA plates were developed with sera from: (1) a pig infected with the Malta isolate of ASFV (this pig was a different animal from the donor of serum MI92 used to screen the library); (2) pigs sequentially infected with non-pathogenic OURT88/3 (day 0) and with OURT88/1 (day 28) and bled 6 days later; and (3) a bush pig that had been infected with the Malawi isolate of the virus and bled 90 days after a secondary challenge with virus.
Fig. 4. (A) Clone 12 encodes a previously unrecognized minor ORF from the virus (C44L). cDNA sequence of minor ORF C44L from the BA71V isolate of ASFV. The positions of the start codon of the minor ORF, the consensus transcription termination signal for ASFV and the poly(A) tail of the cDNA are indicated. (B) Protein gel and Western blot of expressed minor ORF C44L subcloned in pGEX. Protein expression was induced and cell lysates for Western blot analysis were prepared as described in Methods. Samples were analysed on a 10% polyacrylamide gel and then Western blotted as described in Methods. The molecular masses of the proteins identified and the identities of each of the isolated viral cDNAs are indicated. The corresponding Coomassie blue-stained gels of viral ORFs (indicated with an asterisk), subcloned and induced in the pGEX expression system, are also shown. (C) Nucleotide sequence and predicted amino acid sequence of the putative minor viral ORF C44L. The positions of the start of the cDNA, the initiation codon and the ASFV transcription-termination consensus are indicated. Also shown is the predicted amino acid sequence of the minor ORF encoded by the cDNA with internal CAST amino acid repeats highlighted in bold.

All of the expressed proteins recognized by serum MI92 from Malta-infected domestic pigs were recognized by ELISA with immune antiserum from a different domestic pig that had also been infected with the Malta isolate of ASFV (Fig. 5). In addition, the small ribonucleotide reductase subunit/F334L was clearly recognized by ELISA with immune antiserum from this Malta-infected pig (Fig. 5), although it was not identified by Western blot, suggesting that this protein may encode conformational epitope(s) recognized by the antisera used in these experiments.

None of the sera reacted with GST by either Western blot or ELISA (Fig. 3, pGEX vector, and Figs 5A, 6A, GST control). Significantly, all of the recombinant virus proteins were also recognized by immune bush pig serum in both ELISA and Western blot assays (Fig. 6A–C and Table 2). Moreover, with the exception of thymidine kinase/K196R, all of the recombinant proteins were recognized by antiserum from a domestic pig that had been rendered resistant by sequential challenge with the OURT88/3–OURT88/1 virus combination (Fig. 6A–C and Table 2). Apart from thymidine kinase/K196R, antibody responses against all of the purified viral proteins were reproducible in four separate OURT88/3-infected domestic pigs (results not shown). The E184L and K145R viral subunits could not be purified. By Western blot analysis, however, they were recognized by ASFV Malta immune antiserum, although sera from an infected bush pig and OURT88/3–OURT88/1 sequentially infected domestic pigs were negative (Fig. 3).

As this was a preliminary experiment, quantification and reproducibility of these responses to viral antigens must await

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Fig. 5. Recognition of ASFV recombinant proteins by antibodies in sera from outbred domestic pigs experimentally infected with the Malta isolate of ASFV. ELISA plates were sensitized with purified recombinant fusion proteins and then developed with serum from a Malta isolate infected domestic pig collected before and 28 days after infection with the virus. Soluble GST from *S. japonicum* was used to sensitize plates as a negative control. (A) Viral structural proteins, (B) viral non-structural proteins and (C) unassigned viral subunits recognized by antiserum from an immune domestic pig before (open bars) and 28 days after (filled bars) infection with the Malta isolate of ASFV. Values shown are the mean of triplicate observations and SD were less than 5% of the mean. All data shown are a result of experiments performed with 1:160 dilutions of the respective antiserum.

A more systematic study. Nonetheless, sera from both domestic pigs and the bush pig studied were very clearly positive by ELISA when tested against the structural immunodeterminants p73/B646L, p32/CP204L and p10/K78R and the non-assigned subunit CP312R. The observation of clearly positive ELISA titres against these subunits in domestic pigs both 28 days after primary infection with the OURT88/3 isolate and 6 days after secondary challenge with the virulent OURT88/1 isolate is consistent with their possible involvement in protective humoral immunity.

Fig. 6. Recognition of ASFV recombinant proteins by antibodies in sera from domestic pigs sequentially infected and challenged with the OURT88/3 and OURT88/1 isolates of ASFV and in serum from bush pigs infected and challenged with the virulent Malawi SINT90/1 isolate of the virus. ELISA plates were sensitized with purified recombinant fusion proteins and then developed with sera from a domestic pig and bush pig experimentally infected and challenged with the OURT88/3 and OURT88/1 and Malawi SINT90/1 isolates of the virus, respectively, as described in Methods. Soluble GST from *S. japonicum* was used to sensitize ELISA plates as a negative control. (A) Viral structural proteins, (B) viral non-structural proteins and (C) unassigned viral subunits recognized by antiserum from an immune domestic pig before (open bars) and 6 days after (hatched bars) infection with the non-pathogenic OURT88/3 isolate of ASFV, and by antiserum from an immune bush pig before (shaded bars) and 90 days after challenge (filled bars) with the virulent SINT90/1 isolate of ASFV. Values shown are the mean of triplicate observations and SD were less than 5% of the mean. All data shown are the result of experiments performed with 1:160 dilutions of the respective antiserum.
Discussion

There are two models for protective immunity to ASFV: the resistant, natural wildlife hosts, the bush pig or warthog, and the recovered, susceptible domestic pig. The serological response of both of these models has been addressed in this work. Of these two, the former is clearly the most effective and biologically relevant, whilst the second is less effective but economically relevant. At a practical level, of course, the domestic pig is easier to study and so it is hardly surprising that protective immunity has only been studied in this animal, with the definition of immune mechanisms and identification of relevant immunodeterminants as an essential prelude to the rational construction of a vaccine.

It is probable that effective elimination of ASFV requires both serological and cellular immunity. Direct evidence consists of: (1) delayed onset of symptoms in infected pigs that have received a transfusion of recovered pig serum (Ruiz-Gonzalvo et al., 1986; Schlafer et al., 1984); (2) some success in vaccination with the purified viral structural proteins p54 and p32 (Gomez-Puertas et al., 1998); (3) demonstration of cytotoxic T cells in ASFV-infected pigs (Martins et al., 1993; Alonso et al., 1997; Leitão et al., 1998; Jenson et al., 2000); and (4) abrogation of established protective immunity by administration of monoclonal anti-porcine CD8 antibodies in vivo (M. S. Denyer, C. Oura, H. Takamatsu and R. M. E. Parkhouse, unpublished work).

The prime objective achieved in this work is the identification of the principal serological immunodeterminants of ASFV in both the susceptible domestic pig and the resistant wildlife host, the bush pig. This was initially done by antibody screening of a viral cDNA expression library and then confirmed by demonstrating similar reactivities in sera from infected recovered domestic pigs and one infected resistant bush pig. The minor differences observed between the infected bush pig and the domestic pigs may result from differences in virulence and/or immunogenicity of the virus isolates employed.

These results support the notion that there may be a key core of serologically dominant virus immunodeterminants, regardless of the virus isolate (summarized in Table 2), consisting of five structural, three non-structural and six proteins with unassigned functions. It is possible that some of these subunits will prove to play a role in immunity and this is now open to direct experimental testing. Although an effective vaccine relying solely on the stimulation of serological immunity is unlikely to provide effective protection, the definition of protective serological virus immunodeterminants does, nevertheless, provide useful information relevant to the eventual construction of a vaccine.

Clearly, the structural proteins comprising the virus capsid constitute a logical target for protective antibodies and so it may be significant that three of the five structural proteins identified in this study (p32/CP204L, p54/E183L and p73/B646L) have been shown previously to encode important neutralizing epitopes of ASFV (Gomez-Puertas et al., 1996, 1998). Our studies also show that the structural proteins bacterial histone-like protein/A104R and the virus DNA-binding protein/K78R are recognized by antisera from recovered domestic pigs and bush pigs.

Three of the isolated cDNAs encoded non-structural proteins: small subunit of ribonucleotide reductase/F334L, thymidine kinase/K196R and DNA ligase/NP419L. Antibodies to non-protective immunodeterminants of pathogens are a common occurrence in infectious diseases and ASFV is unlikely to be different. A direct role for humoral responses against such non-structural subunits of the virus in protective immunity is not immediately apparent. Without definitive evidence, however, we cannot exclude the possibility that some of the viral enzyme subunits we have isolated are incorporated into the virion, especially as there is already evidence for this in the case of the ASFV ubiquitin-conjugating enzyme (Hingamp et al., 1995). Immunization with non-structural proteins can protect against virus infection (Lin et al., 1998). Indeed, in some cases, incorporation of epitopes from non-structural proteins within multicomponent peptide or DNA vaccines has been demonstrated to be beneficial (Cedillo-Barron et al., 2001). In so far as the presence of antibody against these subunits is an indicator of a helper T cell response, responses against these particular immunodeterminants may be of some relevance, since T cells may also contribute to immunity by providing a source of cytokines to help the generation of antibody against protective immunodeterminants.

Interestingly, three of the six serologically dominant immunodeterminants with unassigned function (C44L, CP312R and E184L) have not been identified previously. The other isolated subunits included B602L, shown previously to be recognized by antisera from convalescent pigs (Irusta et al., 1996), K145R and K205R (Yañez et al., 1995). ORFs K205R and K145R encode early and late proteins of the virus, respectively (Yañez et al., 1995). Viral subunits encoded by ORFs CP312R and K205R were strongly recognized by antisera from immune bush pigs and domestic pigs, raising the possibility that they may be important in the protective antibody response.

In conclusion, these results support the notion that the antibody response against different isolates of ASFV is directed against multiple but shared virus determinants in both the natural bush pig host and domestic pigs and, thus, there may be a key core of serological virus immunodeterminants,
regardless of the virus isolate. Which of the viral subunits we have isolated is involved in the serological component of a protective immune response against the virus awaits further study but the list of viral proteins to investigate is relatively short and the relevant experiments are practically feasible. Immunolocalization and protection studies may permit a characterization of the minimum number of viral subunits necessary for incorporation into a multicomponent vaccine. Alternatively, vaccination with a disabled recombinant virus, as has been reported by Moore et al. (1998), may offer a more successful approach.

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