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An African Swine Fever Virus Gene with Similarity to the T-Lymphocyte Surface Antigen CD2 Mediates Hemadsorption

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An open reading frame, LMW8-DR, in the African swine fever virus (ASFV) genome possesses striking similarity to the lymphocyte membrane antigen CD2. All characterized CD2 domains, including the amino-terminal signal sequence, IgV, hinge, IgC2, stalk, transmembrane, and proline-rich carboxy cytoplasmic domains, are highly conserved in the ASFV gene. Critical residues for the binding of the lymphocyte function-associated antigen (LFA-3) and CD59 and for T-cell activation are also partially conserved. LMW8-DR is actively transcribed in ASFV-infected swine macrophages and Vero cells at late times in the infection cycle and Vero and COS cells transiently expressing the LMW8-DR open reading frame hemadsorbed swine red blood cells. The structural and functional similarities of LMW8-DR to CD2, a protein that is involved in cell–cell adhesion and immune response modulation, suggest a possible role in the pathogenesis of ASFV infection.

African swine fever virus (ASFV), a large icosahedral double-stranded DNA-containing arbovirus, is the causative agent of African swine fever (ASF), an economically significant disease of domestic pigs. ASF occurs in several disease forms, ranging from highly lethal to subclinical infections (*1–3*). ASFV infects cells of the mononuclear-phagocytic system; affected tissues, mainly those of the reticuloendothelial system, show extensive necrosis following infection with highly virulent viral strains (*3, 4*). Moderately virulent ASFV strains also infect these cell types but the degree of tissue involvement and the resulting tissue damage are much less severe (*2, 3*). The abilities of ASFV strains to replicate efficiently and to induce marked cytopathology in these cell types appear to be critical factors in ASFV virulence. Aspects of the virus–cell–host interaction responsible for these differences in viral virulence are unknown.

CD2, a non-polymorphic surface glycoprotein present on all mature T-cells, plays an important physiological role in augmenting the antigen-dependent recognition function of T cells by facilitating cell–cell adhesion (*5, 6*). CD2 is also involved in antigen independent T-cell activation (*7–9*). The natural ligands for CD2 are CD58 (LFA-3), a surface glycoprotein present on most cell types, which is responsible for the rosetting of sheep red blood cells on the surface of T-lymphocytes, and CD59, an erythrocyte protein with complement inhibitory activity (*10*). Here, we describe an ASFV gene, *LMW8-DR*, with significant structural and functional similarity to the lymphocyte adhesion molecule CD2.

A 15.9-kilobase pair (kbp) DNA fragment of the virulent ASFV isolate Malawi Lil-20/1 genome (0.34 to 0.44 MU) contained within the recombinant λ clone LMW8 (*11*) was isolated, subcloned, and sequenced in its entirety using a random sequencing strategy employing the dideoxy-chain termination method and an ABI 370A automated sequencer (*12, 13*). Random sequences were assembled using the computer programs of Staden (*14, 15*). Sequences from both strands were obtained from the LMW8-DR gene region.

LMW8-DR, an open reading frame (ORF) of 1125 base pairs (bp), begins at position 3120 and ends at position 4245 on the positive strand of the 15.9-kbp DNA fragment (Fig. 1). Codon usage bias over an uninterrupted stretch of 1100 codons (95% of the coding region) coincides with the codon frequency of known ASFV genes (*16–19*), suggesting LMW8-DR is protein coding. Additional indications that this region is protein coding include: 12 transcription stop sites immediately preceding the ORF and five stop sites following the ORF; the sequence motif, TAAAATG, which includes the transcription start codon for the ORF, resembles efficient vaccinia virus late promoters (*20, 21*); and three AT-rich nucleotide regions 40, 60, and 100 bp upstream from the start site resemble ASFV promoters (*22*).

The protein predicted by LMW8-DR is 375 amino acids in length, has an isoelectric point of 6.22, and a molecular weight of 42.4 kDa. A potential hydrophobic signal sequence with a cleavage site (*23*) at Ser16 is

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. L15337.

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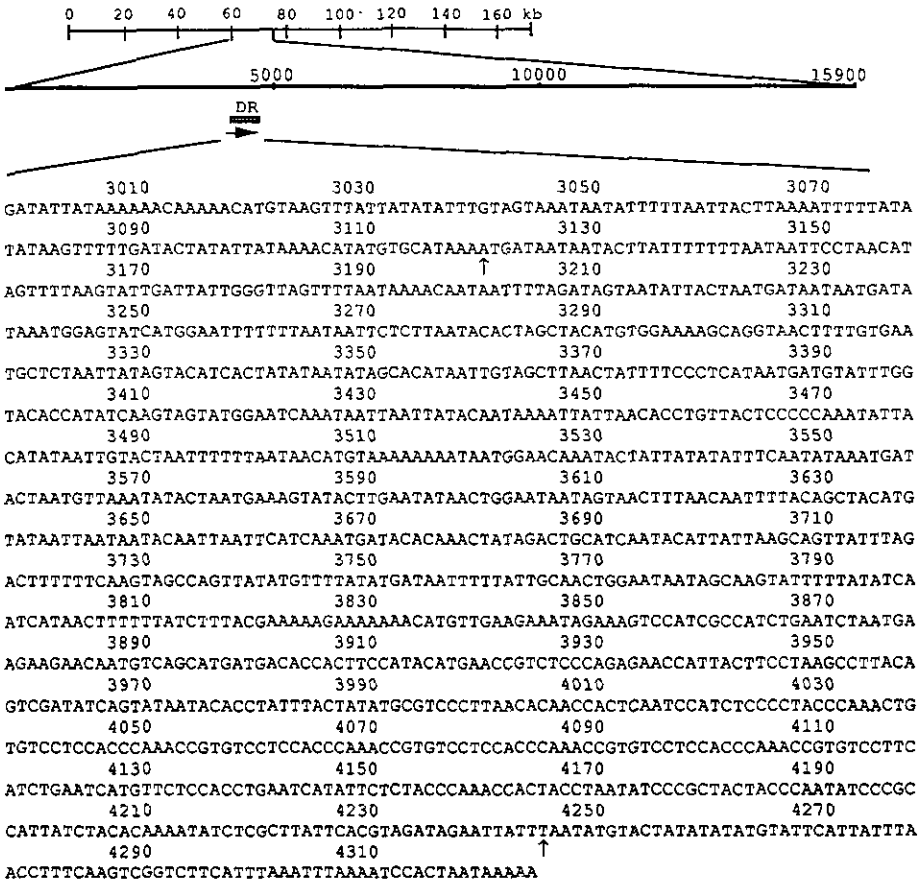
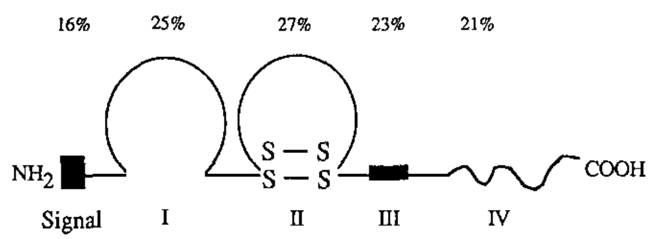


FIG. 1. Nucleotide sequence of ASFV ORF LMW8-DR. LMW8-DR, depicted as the hatched rectangle labeled DR, is located in the left central region of the ASFV (Malawi Lil-20/1) genome in the 15.9-kbp LMW8 λ clone (*11*). An arrow depicts the orientation of the gene. The nucleotide sequence of the coding stand of LMW8-DR is shown here on the positive strand. Numbering is consistent with the location of the gene on the LMW8 λ clone. The open reading frame start and stop sites are indicated with vertical arrows.

located at the amino-terminal region while a potential hydrophobic transmembrane-spanning region occurs from amino acid residue 195 to 230. The carboxy-terminal 145 amino acid stretch is very hydrophilic and proline rich, with the amino acid pattern (CPPPKP) repeated four times starting at residue 307. A search of the Prosite (*24*) database (release 10) identified 15 potential Asn glycosylation sites (*25*) preceding residue 175 and one site at residue 357; four consensus protein kinase C phosphorylation motifs (*26*) at amino acids 101, 123, 224, and 261; and eight casein kinase phosphorylation motifs (*27*) at residues 147, 173, 189, 239, 243, 256, 261, and 330.

FASTA (*28*) and BLAST (*29, 30*) searches of GenPept (release 75), Swiss-Prot (release 25), PIR (release 36), Kabet immunological (release 4-7-93), MIPS (release 34), and TFDAA (release 6.0) databases as well as TFASTA and TBLASTN searches of GenBank (release 75) and EMBL (release 34) revealed a striking similarity between LMW8-DR and the cell adhesion molecule CD2 of human (*31–33*) (FASTA = 209, z = 22.4), rat (FASTA 174, z = 19.1), and mouse (FASTA = 152, z = 14.5). As with CD2, database searches with LMW8-DR did not reveal any similarity when directed at other members of the immunoglobulin superfamily. LMW8-DR (Fig. 2) is similar in size and amino acid composition to mouse, rat, and human CD2 (38 kDa). There is a potential amino-terminal signal of approximately 20 residues. The following 180 residues of LMW8-DR correspond to the extracellular portion of CD2 and contain a domain (DI) with limited similarity to the CD2 IgV-like domain, a hinge domain at Val114, a domain (DII) with similarity with the CD2 IgC2-like domain, and a stalk at Cys190. As in the CD2 molecule, LMW8-DR has a central potential hydrophobic transmembrane region followed by a basic, proline-rich cytoplasmic carboxy-terminal region of 150 residues (*34*). Comparing the entire sequence of LMW8-DR to human CD2, there is a 22% identity and a 37% conservation of amino acids based on the Dayhoff PAM-250 symbol comparison table with a 0.5 cutoff (*35*). Even more striking are the 25% identity and 43% conservation over the 180 amino acids of the LMW8-DR corresponding to the human CD2 extracellular Ig-like domains (Fasta 150, z = 17.1). In fact, for the LMW8-DR domain (DII), corresponding to the CD2 IgC2-like domain, the canonical

A.



B.

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                  *          IA      * *                                            * *          *    IB
RAT     29 WgaLghgInLNipnfqmtDDIDEVrWE..rgS..TLvAef.KrkmkpFlk  74 SgaFEIlaNgdLkIkn.LtRDDsgtynVtv..YstngtrILDkaLdLRIL
MOUSE   29 WgvLghgItLNipnfqmtDDIDEVrWv..rrg..TLvAef.KrkkppFli  74 SetYEVlaNgSLkIkkpMmRNDsgtynVMv..YgtngmtrLEkdLdVRIL
HUMAN   34 WgaLgqdInLDipsfqmsDDIDDIkWE..ktSdkkkiAqfrKekEt.FkE  81 kdtYkLfkNgtLkIkh.LktDDqdiykVsi..YdtkgknVLEkiFdLKIq
LMW8DR  20 WvsFnktIiLDsnitndnNDINGVsWNffnnSlnTL.AtcgKagN..FcE  73 Sl.YNIahNcSLtI...FpHND.....VFgtpYqvvwnqIIN..YtIKLL

                                       R1                                                                 R2

                  *     **                          II      *           *    *               *
RAT    123 VskPmIyWECsNatLTCevlEGTDveLkLYq.gkE.HLRslrQktMsYqWtN.lrapFk..CkavNrVsqESemEv..VNCpEkgLp
MOUSE  124 VskPmIhWECpNttLTCavlQGTDfeLkLYq.g.ETllnslpQknMsYqWtN.lnapFk..CeaiNpVskESkmEv..VNCpEkgLS
HUMAN  130 VskPkIsWtCiNttLTCevmNGTDpeLnLYqDgk..HLK.lsQrVIthkWttSlsakFk..CtagNkVskESSvEp..VsCpEkgLd
LMW8DR 114 VtpPnItYNCtNflITCkknNGTN.tI.IYFNinDTNVKytnEsILeYnWnNSnfnnFtatCiinNtI..NSSnDtqtIDCiNtlLS

                                    R3

                  III                              IVA                        IVB
RAT    204 YL.IVgVsAggLlLVFFgA......LFIfcIc  229 KRKKRnrrrkgEELEikasrmStvE  254 RGPkPHstqasaPaS..Q
MOUSE  205 YV.tVgVgAggLlLVLLVA......LFIfcIc  230 KRRKRnrrrkdEELEikasrtStvE  255 RGPkPHst....PaaaaQ
HUMAN  211 YL.IIgIcGggslLMVFVA......LLVfyIT  236 KRKKQrsrrndEELEtrahrvatEE  261 RGrkPqQ....iPaStpQ
LMW8DR 198 YLdFFqVaSymFyMIIFIAtgiiasIFIsiIT  235 KRKKH.....vEEIEspspseSnEE  266 HEPsPREpllpkPySryQ

                                   IVC                                        IVD
RAT    .271 PVasQ.apPpPg.hhlqtPghrPlPpshrnrehqpkkRPpPSGtqvhqqkGppLPRP    327 VqPkpPcgSgD.VSL
MOUSE  .270 sValQ.apPpPg.hhlqtPghrPlPpghrtrehqqkkRPpPSGtqihqqkGppLPRP    326 VqPkpPcgSgDgVSL
HUMAN  .276 PaTsQhppPpPg.hrSqAPshrPPPpghrvqhqpqkrpPaPSGtqvhqqkGppLPRP    333 VqPkpPhgaaEN.SL
LMW8DR.293 PLT.QplnPsPlpklCpPPkpcPPPkpcpppkpcpppKPcPSSescsppeSysLPKP    354 LlPniPplStQNISL
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FIG. 2. (A) Comparison between LMW8-DR domains (I, II, III, IV) and CD2 domains: signal sequence, IgV-like domain (Ia, b), IgC2-like domain (II), transmembrane region (III), cytoplasmic proline-rich region (IVa, b, c, and d). Percentage amino acid identities of LMW8-DR to human CD2 domains are listed above the diagram. (B) Comparison between the amino acid sequence alignments of LMW8-DR with human (*31*), mouse (*33*), and rat (*32*) CD2. Amino acid identities with LMW8-DR are in bold uppercase letters while conservative amino acid substitutions are in uppercase letters. The numbering of LMW8-DR is consistent with the amino acid sequence shown in Fig. 1. The other proteins are numbered as they appear in the citations. The three functional domains of human CD2 (R1, R2, and R3) associated with CD58 and CD59 binding and with T-cell activation are labeled and underlined. Asterisks mark the important conserved residues of domain I, lacking the typical Ig disulfide bond as well as domain II, containing the typical Ig disulfide bond.

pair of disulfide bonds C123, C190, and C130, C173 with intervening conserved residues Thr129, Trp161, and Asn177, exactly line up with CD2 and are the same distance apart (*36*). The LMW8-DR domain DI, corresponding to the CD2 IgV-like domain, has no disulfide bond in contrast to the conventional IgV domains which contain one disulfide bond. In particular, the LMW8-DR domain D1, contains the conserved amino acid residues Leu29, Val43, Trp45, Leu83, Ile85, and Asp90 found in CD2 and other Ig molecules containing this domain laking the disulfide bonds (*36*). Other areas of similarity between LMW8-DR and CD2 include the potential transmembrane region (23% identity and 51% conservation over 35 amino acids).

The domains DI and DII of LMW8-DR, corresponding to the CD2 Ig-like domains, also exhibit conservation in the three functionally active domains of the human CD2 (Fig. 2), which are involved in CD58 and CD59 binding (*10, 37–39*) and T cell activation (*2, 7–9, 37, 39*). LMW8-DR residues Phe47 to Ala61 correspond to the human CD2-CD58 binding and T-cell activation region I (Lys65 to Phe71), with three of the seven important amino acids conserved (Thr54, Leu55 and Ala56) in LMW8-DR. LMW8-DR residues Tyr96 to Ile104 correspond to the CD2-CD58 and CD-59 binding region II (Tyr110 to Leu118) with one identity (Tyr96) and two of the seven important amino acids conserved (Ile103 and Ile104) in LMW8-DR. LMW8-DR residues Tyr141 and Phe142 correspond to the CD2 T-cell activation region III (Tyr159–Gln160) with the Tyr141 being identical in LMW8-DR.

The main differences between LMW8-DR and CD2 include the presence in LMW8-DR of the amino acid repeat CPPPPCK and two insertions, of 11 and 9 amino acids each, in the carboxy end, which are not present in CD2. Also, LMW8-DR contains 15 potential

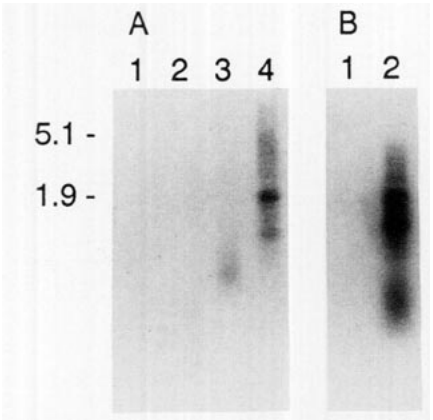

FIG. 3. Northern analysis of LMW8-DR in ASFV-infected cells. Late expression of LMW8-DR was observed in infected macrophages (A) and Vero cell lines (B) infected at high multiplicity (m.o.i. = 20) with the virulent E-75 (Spain) isolate, or its low passage Vero adapted varient, E-75 CV5, respectively. Total cell RNA was prepared and analyzed as previously described (*55, 56*). RNA samples (10 μg) were treated with RNase free DNase I (1 μg/ml) and hybridized [0.5 *M* $Na_2H_2PO_4$ (pH 7.2), 1% bovine serum albumin, 1 m*M* EDTA, 7% SDS] at 65° with a single-stranded ^{32}P-labeled DNA probe complementary to the LMW8-DR ORF coding region (*57*). RNA from mock-infected (lane 1) or infected macrophages was isolated at 3 hr (lane 2), 6 hr (lane 3), and 24 hr (lane 4) postinfection. RNA from Vero cells infected in the presence (lane 1) or in the absence (lane 2) of ARA-C was collected at 15 hr postinfection. Size markers are in kilobases.

Asn glycosylation sites in the Ig-like domains, whereas CD2 contains only three or four Asn glycosylation sites.

The only other similarity found in database searches was between the potential transmembrane region of LMW8-DR and the third transmembrane region of the acetylcholine receptor (32% identity and 67% conservation over 31 amino acids). Also, the LMW8-DR carboxy-terminal region starting at residue 331 was similar to the cytoplasmic region of the acetylcholine receptor beginning at residue 420 (32% identity and 42% conservation over 31 amino acids).

LMW8-DR is transcribed at late times in ASFV-infected cells (Fig. 3). Northern blot analysis demonstrated the presence of LMW8-DR transcripts in swine macrophages at late but not at early times postinfection. Additionally, LMW8-DR transcripts were not detected in ASFV-infected Vero cells in the presence of cytosine arabinoside, a drug which inhibits viral DNA replication and late gene expression (*40*). Similar to vaccinia virus late RNAs, LMW8-DR transcripts were heterogeneous in size (*41, 42*).

Given that CD2, a protein present on the surface of T lymphocytes, is responsible for rosette formation with sheep red blood cells (*5*) and that hemadsorption of swine red blood cells (SwRBC) to ASFV-infected cells is observed with most ASFV isolates (*43, 44*), it is possible that LMW8-DR may mediate hemadsorption to virus-infected cells. To examine this question, the LMW8-DR gene was cloned and then expressed in Vero and COS cells using a vaccinia virus (VV) T7 promoter-driven transient expression system (*45*). The complete LMW8-DR ORF was amplified by polymerase chain reaction (PCR) from the ASFV λ clone LMW8 (*11*) using a primer pair that introduced a *Sal*I and *Xho*I restriction site at the 5′ and 3′ ends of the gene, respectively (forward primer 5′-AAAACATATGTCGACAAA-ATGATAA and reverse primer: 5′-ATGTTCTCGAGG-ATCTGCTACTA). The PCR product was then cloned into the *Sal*I/*Xho*I site of pET21b (Novagen, Madison, WI). Using this cloning strategy, the first 20 amino acids of the vector T7 polymerase tag sequence remain intact and are translated in frame with the cloned LMW8-DR ORF. The amino terminus of the resulting clone, pET-CD2/1, was sequenced to ensure fidelity of sequence amplification and proper framing of the LMW8-DR ORF (data not shown). To serve as a control, the ASFV gene LMW23-NL (*46*) was amplified by PCR and cloned into the *Nde*I site of pET 21. Because of the cloning site used, this expression construct, pET-NL/1, lacks the vector T7 polymerase tag sequence.

Transient expression of the cloned LMW8-DR ORF (pET-CD2/1) in COS cells is shown in Fig. 4. A novel protein of the expected molecular weight of 40–42 kDa was immunoprecipitated with an anti-T7 polymerase tag mAb (Novagen) from COS cells transfected with the pet-CD2/1 construct (T7 tag +) but not from cells expressing the ASFV ORF LMW23-NL (T7 tag −) or

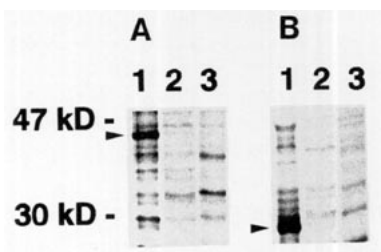

FIG. 4. Transient expression of ASFV ORFs LMW8-DR and LMW23-NL in COS cells. Expression was obtained using a vaccinia virus T7 promoter driven expression system (*45*). COS cells at 30–50% confluency were infected (m.o.i. = 1–5) with the recombinant vaccinia virus VTF-3 containing the T7 polymerase gene (*45*). At 3 hr PI the non-adsorbed virus was removed, cells were washed three times with Optimem (Gibco-BRL, Gaithersburg, MD), and transfected with 10 μg of cesium–chloride gradient purified plasmid DNA using Lipofectamine (Gibco-BRL). ASVF ORFs, LMW8-DR and LMW23-NL, were cloned in frame into the T7 expression plasmid pET21b (Novagen, Madison, WI). Immunoprecipitations of cell lysates were performed as described previously (*58*). (A) Immunoprecipitation of LMW8-DR with an anti-T7 polymerase tag mAb: Lanes 1, LMW8-DR expressing cells; 2, LMW23-NL expressing cells; 3, mock-transfected cells. (B) Immunoprecipitation of LMW23-NL with monospecific anti-NL antibody (Afonso *et al.*, unpublished results): Lanes 1, LMW23-NL expressing cells; 2, LMW23-NL expressing cells immunoprecipitated with preimmune serum; 3, mock-transfected cells immunoprecipitated with anti-NL antibody.

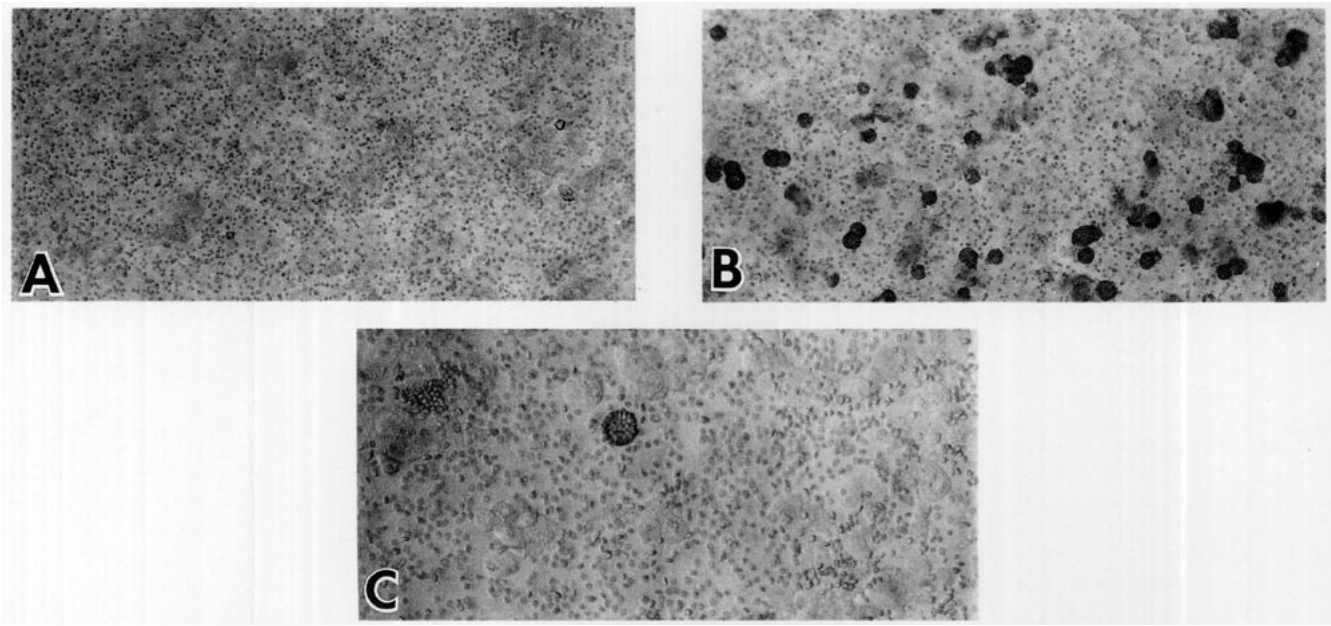

FIG. 5. Hemadsorption of SwRBC to Vero cells transiently expressing ASFV ORF LMW8-DR. ASFV genes were transiently expressed in Vero cells as described in Fig. 4. Infected/transfected cells were collected by gentle scrapping at 48 hr p.i., reseeded into 96-well plates, and immediately mixed with 40 μl of a 2% suspension of SwRBC. Rosette formation was scored 24 hr later. (A) Cells expressing a control ASFV gene, LMW23-NL; (B, C), cells expressing LMW8-DR. Note the extensive rosette formation present in this expressing cell culture. Magnifications: (A, B) ~56×, (C) ~113×.

from mock-transfected VV-infected COS cells (Fig. 4A, lanes 1–3, respectively).

Both Vero and COS cell transiently expressing LMW8-DR formed specific rosettes with SwRBC (Figs. 5B and 5C). Rosette formation was not observed in any control cultures, which included: Vero cells transiently expressing the AFSV gene LMW23-NL (*46;* Fig. 4B, Fig. 5A); non-transfected, VV-infected VERO, and COS cells; and COS cells expressing the human LFA-3 gene (data not shown). Thus, the protein product of LMW8-DR alone is sufficient for mediating SwRBC hemadsorption. This observation suggests that LMW8-DR alone may be responsible for the hemadsorptive characteristic of AFSV infected cells (*43, 44*).

Although the function of LMW8-DR in ASFV infection and immunity is unknown, its structural and functional similarities with CD2, a gene involved in cell–cell adhesion and T-cell-mediated immune responses, suggest a possible, but as yet unclear, role for it in aspects of ASFV pathogenesis. Although non-hemadsorbing ASFV isolates with reduced virulence for pigs have been described (*47, 48*), there is not a direct correlation between hemadsorption and pig virulence; relatively avirulent hemadsorbing isolates and non-hemadsorbing virulent ASFV isolates exist (*49, 50*). Interestingly, and of possible significance for aspects of ASFV pathogenesis, the viremia observed in pigs following infection with high virulent and moderately virulent ASFV isolates is associated predominately with the erythrocyte fraction (*51–54*). The virus protein(s) mediating this virus–erythrocyte interaction has not been described, although it appears that the outer membrane of the virion is important for this interaction (*48*). LMW8-DR may be involved in some aspect of this, or perhaps in virion attachment and penetration to other specific cell types. It is also reasonable to suggest, given these structural and functional similarities with CD2, that LMW8-DR may function in altering normal T-cell-related effector mechanisms in the infected host.

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