INTRODUCTION

Yaba-like disease virus (YLDV) belongs to the genus Yata-poxvirus of the subfamily Chordopoxvirinae and causes vesicular skin lesions in primates (Knight et al., 1989), although the natural reservoir of this virus is uncertain. Like other poxviruses, the genome of YLDV has a central conserved region and more variable terminal regions encoding genes that might affect virulence, host range or immune evasion (Lee et al., 2001). Only a few YLDV genes have been characterized. YLDV protein 134R is a cytokine related to interleukin (IL)-19, IL-20 and IL-24 that binds to the IL-20 receptor type 1 (IL-20R, interleukin (IL)-19, IL-20 and IL-24 receptor) (Lee et al., 2001). Only a few YLDV genes have been identified. YLDV 7L also bound CCL1, with high affinity ($K_d = 0.6 \text{ nM}$) and induces signal transduction by activation of heterotrimeric G proteins and downstream protein kinases. Further characterization of YLDV 7L is presented here and shows that murine CC chemokines can induce G-protein activation via the 7L receptor, despite having a low binding affinity for this receptor. In addition, when expressed by recombinant vaccinia virus (VACV), YLDV 7L was found on the outer envelope of VACV extracellular enveloped virus. The contribution of 7L to poxvirus pathogenesis was investigated by infection of mice with a recombinant VACV expressing 7L (vB8R-7L) and was compared with the outcome of infection by parental and revertant control viruses. In both intranasal and intradermal models, expression of 7L caused attenuation of VACV. The role of this protein in viral virulence is discussed.

There are several interesting features of the interaction between CCR8 and CCL1. First, although many chemokine receptors can bind multiple ligands, CCL1 is the only host ligand that binds CCR8 with high affinity (Tiffany et al., 1997). The only other ligands for CCR8 that have been identified are vMIP-I and vMIP-II from HHV-8 (Dairaghi et al., 1999). Second, CCR8 is expressed by monocytes, dendritic cells (DCs) and different subsets of T cells (D’Ambrosio et al., 1998; Colantonio et al., 2002; Qu et al., 2004) and its interaction with CCL1 is important for the positive selection of thymocytes (Kremer et al., 2001), skin homeostasis (Schaerli et al., 2004) and migration of DCs to lymph nodes (LNs) during the inflammatory response (Gombert et al., 2005). Hence, the CCR8–CCL1 interaction functions in housekeeping (development and homeostasis) and inflammation (generation of innate and adaptive immune responses). Third, several viruses have targeted the CCR8–CCL1 interaction, either by expressing proteins related to CCR8 or by expressing chemokines that bind to CCR8. In addition to YLDV, other chordopoxviruses (capripoxvirus, suipoxvirus and deerpox virus) encode CCR8-like proteins, although these have not yet been characterized. Collectively, these observations suggest that the CCR8–CCL1 interaction plays an important role(s) during virus infection.

Viral chemokine receptors (vCKRs) are encoded by both poxviruses (http://athena.bioc.uvic.ca/ca.virology/php/) and herpesviruses and several roles have been proposed for these...
molecules in vivo (Alcami, 2003). If the vCKR can induce signal transduction (inducible or constitutive), it may modify cell physiology to the virus’ advantage (Arvanitakis et al., 1997). Alternatively, binding a ligand with high affinity (with or without signalling) can deplete chemokines from the extracellular medium (Bodaghi et al., 1998). It is also possible that a vCKR might aid viral dissemination by causing the infected cell to migrate along a chemokine gradient. Lastly, incorporation of vCKRs into the virus envelope might affect the initial steps of virus replication (Frale-Ramos et al., 2002).

Genes acquired by viruses from their host may be modified during evolution to the advantage of the virus. With vCKRs, alterations in the extracellular N-terminal region and loops connecting transmembrane domains may affect ligand binding (Casarosa et al., 2005). Similarly, changes within the intracellular C-terminal domain and loops connecting transmembrane domains may affect internalization and signal transduction. For instance, alterations in the conserved DRY motif can affect signalling (Rosenkilde et al., 2005) and changes within the transmembrane domains can influence receptor dimerization (Burger et al., 1999). Lastly, alteration of existing motifs or generation of new motifs that induce post-translational modifications (phosphorylation, sulphation or glycosylation) can change the protein functionally (Gutiérrez et al., 2004; Casarosa et al., 2005).

This study provides a further characterization of YLDV 7L. We show that 7L responds to binding of murine chemokines, is incorporated into the vaccinia virus (VACV) EEV and may affect the initial steps of virus replication (Fraile-Ramos et al., 2002).

METHODS

Cells and viruses. Cell lines and viruses were described previously (Najarro et al., 2003).

Reagents. Radiodinated $^{125}$I-hCCL1 [2200 μCi (8-14 MBq) mmol$^{-1}$] was purchased from Du-Pont-NEN and $^{35}$S-GTP-$\gamma$-S [1000 μCi (37 MBq) mmol$^{-1}$] was from Amersham Biosciences. Recombinant human, murine and viral chemokines were from Peprotech, except for mCCL1/TCA-3 and mVMP-I, which were purchased from R&D Systems.

Recombinant virus construction. For in vivo studies, a set of recombinant viruses was constructed in which the 7L gene was inserted into VACV at the B8R gene locus driven by the early/late 7.5K promoter. Gene 7L was excised from pSC11-7L by digestion with SmaI and ligated into pGS15 (Mackett et al., 1984) that had been digested with SmaI, to form pGS15-7L. pGS15-7L was digested with SalI and XbaI to release the VACV p7.5 promoter and 7L and this fragment was ligated into pAB8R that had been digested with SmaI to form pAB8R-7L. pAB8R-7L was used to insert 7L into the deleted B8R region of vAB8R (Symons et al., 2002), using the Ecogpt gene as a selectable marker by transient dominant selection (Falkner & Moss, 1990). The parental virus vAB8R-wt and a revertant virus, vAB8R-rev, from which the inserted 7L was deleted, were also isolated as controls. All virus isolates were plaque-purified and their genomic structures were confirmed by PCR.

RESULTS

Human, viral and murine chemokines act as agonists for 7L

Previously, cells infected with recombinant VACVs expressing 7L were used to identify binding of human and murine chemokines to 7L (Najarro et al., 2003). Here, we have studied chemokine binding by measuring G protein–coupled receptor (GPCR) activation indicated by $^{35}$S-GTP-$\gamma$-S incorporation (Najarro et al., 2003). During GPCR activation, GPCRs exist in equilibrium between an inactive (R) and an active ($R^*$) state. The R to $R^*$ isomerization can occur without ligand binding, resulting in GPCR constitutive activity (reviewed by Seifert & Wenzel-Seifert, 2002). Full and partial agonists stabilize the $R^*$ state of GPCRs and, accordingly, increase GDP–GTP exchange, whereas inverse agonists stabilize the R state of GPCRs and reduce basal GDP–GTP exchange. Finally, antagonists do not change basal G-protein activity, but their binding will block both the stimulatory effects of agonist and the inhibitory effects of inverse agonist. Chemokines CCL1/TCA-3, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5 RANTES, CCL7/MCP-3, CCL16/
LEC, CCL17/TARC, CCL21/exodus-2 and CCL24/eotaxin-2 were tested for their ability to modify 7L basal activity by using membranes isolated from cells infected with a recombinant VACV expressing 7L. Human and murine CCL1, CCL4, CCL7, vMIP-I and vMIP-II caused an increase from basal level, indicating that they are agonists for 7L. In contrast, hCCL3 (MIP-1α), hCCL5, mCCL5, hCCL17, hCCL24 and mCCL24 (eotaxin-2) induced little change (Fig. 1a). This might indicate either lack of binding to the receptor or antagonist action upon 7L. hCCL16 (LEC) caused reduction of bound \(^{35}\text{S}\)GTP-γ-S, suggesting that it was an inverse agonist for 7L. Displacement curves measuring GDP–GTP exchange at increasing concentrations of murine chemokines (Fig. 1b) confirmed the action of murine ligands mCCL1, mCCL3 mCCL4, mCCL7 and mCCL21 as agonists for 7L.

The ability of several murine chemokines to induce G-protein activity via 7L as determined by binding of \(^{35}\text{S}\)GTP-γ-S (Fig. 1a, b) contrasted with the inability of 100-fold excess of these chemokines to displace binding of \(^{125}\text{I}\)hCCL1 to 7L (Najarro et al., 2003). This discrepancy was investigated by additional binding and displacement experiments to generate sigmoid curves (Fig. 2). This showed that mCCL1, mCCL3 and mCCL4 can partially displace the binding of \(^{125}\text{I}\)hCCL1 to 7L. However, the IC\(_{50}\) values for mCCL1 and mCCL4 were one order of magnitude greater than that for hCCL1, and the IC\(_{50}\) for mCCL3 was 1000-fold greater than that for hCCL1. In summary, mCCL1, mCCL3 and mCCL4 bind 7L with low affinity, but nonetheless induce a functional response, as indicated by the GDP–GTP-exchange assay.

7L is incorporated into VACV extracellular virus particles

The outer envelope of VACV cell-associated envelope virus (CEV) and extracellular enveloped virus (EEV) is derived from internal cell membranes through which cell-surface proteins pass during transport to the cell surface (Smith et al., 2002). Accordingly, some cellular proteins derived from Golgi or post-Golgi membranes have been found in CEV/EEV particles (Vanderplasschen et al., 1998; Krauss et al., 2002). To investigate whether 7L, 145R and hCCR8 expressed from VACV were incorporated into CEV/EEV, ultrathin sections of cells infected with v\(\Delta\)B8Rtk–, v\(\Delta\)B8Rtk-7LHA\(_{\text{N}}\), v\(\Delta\)B8Rtk-145RHA\(_{\text{N}}\) or v\(\Delta\)B8Rtk-hCCR8HA\(_{\text{N}}\) were analysed by immunoelectron microscopy using an anti-HA

![Fig. 1. G-protein activation by chemokines.](http://vir.sgmjournals.org)

(a) Membranes from cells expressing 7L were incubated with different chemokines at a final concentration of 200 nM and GDP–GTP exchange was measured after addition of \(^{35}\text{S}\)GTP-γ-S. (b) Measurement of \(^{35}\text{S}\)GTP-γ-S binding with increasing amounts of murine chemokines. Data represent means±SD of determinations in triplicate from a single experiment that was performed two or three times. The basal level of \(^{35}\text{S}\)GTP-γ-S binding is indicated by the dashed lines.

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Yaba-like disease virus chemokine receptor 7L
mAb as described previously (Krauss et al., 2002) and the number of gold particles in CEV/EEV particles (Fig. 3) was counted (Table 1). Gold particles were found in the plasma membrane and outer envelope of CEV/EEV from each virus expressing an HA-tagged seven-transmembrane (7TM) protein. Statistical analysis of the number of gold particles per 100 CEV/EEV particles for each virus expressing a 7TM protein, compared with the vDB8Rtk2 control, showed that each 7TM protein was present in CEV/EEV (Table 1). No gold particles were detected when the primary mAb (anti-HA) was omitted.

**i.n. infection of mice with VACV expressing 7L**

In the absence of a rodent model to study YLDV pathogenesis, the role of YLDV 7L in vivo was studied by using recombinant VACVs expressing 7L. The 7L gene was expressed from the 7.5K promoter and inserted into the B8R locus of vDB8R (Symons et al., 2002). The virulence of this virus (vDB8R-7L) was compared with that of the parent (vDB8R-wt) and revertant (vDB8R-rev) viruses by i.n. infection of BALB/c mice and measurement of signs of illness (Fig. 4a) and weight change (Fig. 4b) as described previously (Alcami & Smith, 1992). Whereas infection with vDB8R-wt or vDB8R-rev induced weight loss from day 3 (maximum, 15 % loss by day 7) and signs of illness from day 4 (maximum score, 2-3 on day 7), animals infected with vΔB8R-7L showed little weight loss (<5 %) and signs of illness (maximum score, 0-25) and had recovered completely by day 8. In a second experiment, the titres of virus in the lungs (Fig. 4c) and the cell infiltration into this organ (Fig. 4d) were measured at days 3, 6 and 9 post-infection (p.i.). On days 6 and 9 p.i., virus titres were reduced significantly after infection with vΔB8R-7L compared with controls. Additionally, on day 6, there was enhanced cellular infiltration of lungs in vΔB8R-7L-infected animals. Analysis of different cell populations indicated that, for vΔB8R-7L, there was a slight reduction in CD3+ cells on day 3 and an increased number of granulocytes (Ly-6G+) on day 6 (data not shown).

The virulence of recombinant VACVs expressing 7L from the tk locus was also examined. VACV mutants lacking TK are attenuated in mice after different routes of infection (Buller et al., 1985; Lee et al., 1992) and more than a 1000-fold increase in LD50 was noted for TK2 mutants after i.n. infection (LD50 > 108 p.f.u.) (Lee et al., 1992). Accordingly, the virus dose administered i.n. was increased to 107 p.f.u.

**Table 1. Gold particles per EEV/CEV section**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mean</th>
<th>95% confidence interval</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>vDB8Rtk−</td>
<td>0</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>vDB8Rtk-7LHA&lt;sub&gt;N&lt;/sub&gt;</td>
<td>2.09</td>
<td>1.32–2.85</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>vΔB8Rtk-145RHA&lt;sub&gt;N&lt;/sub&gt;</td>
<td>1.87</td>
<td>1.1–2.63</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>vΔB8Rtk-hCCR8HA&lt;sub&gt;N&lt;/sub&gt;</td>
<td>2.1</td>
<td>1.33–2.86</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
After infection with vΔB8Rtk⁻, vΔB8Rtk-7L or vΔB8R-7LHAN, all groups exhibited weight loss (approx. 25% by day 7), but there was no significant difference between groups (data not shown). Similarly, at lower doses (4 × 10⁶ and 3 × 10⁵), there were also no differences between the groups (data not shown). These data suggest that tk⁻ VACV mutants are less suitable vectors for expression of potential immunomodulators because their attenuated phenotype can mask changes seen with other mutants.

### i.d. infection of mice with VACV expressing 7L

The virulence of vΔB8R-wt, vΔB8R-7L and vΔB8R-rev was also investigated in an i.d. mouse model (Tscharke & Smith, 1999; Tscharke et al., 2002) (Fig. 5). In this model, also, vΔB8R-7L was attenuated compared with controls and produced significantly smaller lesions that healed more rapidly. The differences observed were significant (P<0.05) from day 8. A noticeable difference in vΔB8R-7L was enhanced redness at the inoculation site before lesion development (day 3 and 4 p.i.) compared with control groups (data not shown). Analysis of the cell populations present in the infected ears and the draining LNs at days 3, 5 and 8 p.i. revealed no obvious differences (data not shown).

![Fig. 4. i.n. infection with VACV expressing 7L. Mice were infected with 5 × 10³ p.f.u. vΔB8R-wt (●), vΔB8R-7L (▼) or vΔB8R-rev (■) and their signs of illness and body weights were recorded daily. Data are presented as (a) mean (± SEM) signs of illness for each group and (b) mean (± SEM) body weight of the animals in the group compared with their respective weight on the day of infection. (c) Mean viral titres in the lungs of mice infected with vΔB8R-wt (empty bars), vΔB8R-7L (filled bars) or vΔB8R-rev (checked bars) at days 3, 6 and 9 p.i. (d) Number of cells recruited to the lungs and present in the bronchoalveolar lavages (BALs) of infected mice. Colour coding as in (c); asterisks indicate P<0.05.](http://vir.sgmjournals.org)

![Fig. 5. Virulence of VACV expressing 7L in the i.d. model. BALB/c mice were injected in the ears with 10 μl (10⁶ p.f.u. per ear) of vΔB8R-wt (●), vΔB8R-7L (▲) or vΔB8R-rev (■) and lesion sizes were estimated daily. Points represent the mean ± SEM lesion diameter.](http://vir.sgmjournals.org)
In summary, vAB8R-7L was attenuated in the i.n. and i.d. models compared with controls.

**DISCUSSION**

YLDV 7L was the first poxviral 7TM chemokine receptor shown to bind chemokines and signal in infected cells. Here, a further characterization of 7L is presented, together with studies of its effect on VACV infection *in vivo*.

The ability of additional chemokines to bind and activate 7L was studied by using \(^{[35}S\)GTP-γ-S in a GDP-exchange assay. This showed that some murine chemokines were able to stimulate GDP–GTP exchange through the 7L CKR, despite not displacing \(^{125}\)IhCCL1 bound to cells expressing 7L (Najarro *et al.*, 2003). This might be explained by the fact that agonists can achieve maximal guanine-nucleotide exchange by submaximal receptor occupancy and each activated receptor can catalyse GDP exchange at more than one G protein. Although the low-affinity murine chemokines probably have a high dissociation constant for 7L, this might not prevent activation of 7L in the GDP-exchange assay, where agonists at concentrations lower than those required for half-maximal receptor occupancy can generate the formation of a ternary complex and binding of \(^{[35}S\)GTP-γ-S (Seifert & Wenzel-Seifert, 2002). The potency of the murine chemokines lay within reported physiological ranges. The different concentrations at which the chemokine receptor-bearing cells can react is broad and the responses will be determined by the strength of the stimuli (Petkovic *et al.*, 2004).

The topology of 7L was established previously by immunofluorescence and showed that the N terminus was exposed on the cell surface (Najarro *et al.*, 2003). This was confirmed here by immunoelectron microscopy, and this analysis also showed that 7L is present in the CEV/EEV outer membrane. Similar observations were made for 145R and CCR8. The implications of these observations are uncertain. On the one hand, 7L would be able to bind CCL1 from this location, but, on the other hand, during virus entry, the 7L protein would not be inserted into the cell membrane because the EEV membrane is lost by a non-fusogenic mechanism that leaves it outside the cell (M. Law, G. C. Carter, K. L. Roberts, M. Hollinhead & G. L. Smith, unpublished data).

Expression of 7L from VACV caused attenuation in both the i.d. and i.n. models of infection. Although, at first glance, the attenuation induced by 7L expression might seem counter-intuitive, this observation is in agreement with expression of a secreted CC chemokine-binding protein from VACV (Reading *et al.*, 2003). It is unlikely, however, that the mechanism of action of 7L is akin to that of soluble binding proteins, which can modulate chemokine activity throughout the infected lung and are not restricted to virus-infected cells (Reading *et al.*, 2003). Possibly, expression of 7L affects the activation state of cells and their ability to recruit or stimulate other cells.

The outcome of insertion of gene 7L into the VACV genome was influenced by the site chosen. When 7L was inserted into the B8R locus, there was a reduction in virulence, but when inserted into the TK gene, there was not. Several factors might explain this. First, knocking out the B8R or TK gene has different effects on VACV virulence in the mouse. Deletion of the B8R gene, which encodes a gamma interferon (IFN-γ)-binding protein (Alcamí & Smith, 1995), did not affect virus virulence in mice (Symons *et al.*, 2002) and this observation was consistent with the fact that the B8R protein does not bind or neutralize mouse IFN-γ (Alcamí & Smith, 1995; Symons *et al.*, 2002). So, in the background of a virulent virus, 7L expression causes attenuation. On the other hand, inactivation of TK causes severe attenuation and, in the mouse i.n. model, the LD<sub>50</sub> of TK<sup>−</sup> viruses was increased by 1000-fold compared with the control (Lee *et al.*, 1992). In the background of attenuated viruses, where 1000-fold-higher doses of virus must be administered to induce comparable disease, the expression of 7L or related CKRs did not affect virus virulence. Second, the insertion site can affect expression levels of the foreign gene (Bennett *et al.*, 1999; Coupar *et al.*, 2000). Third, it is possible that the expression of 7L from the B8R, but not TK, locus affects neighbouring genes adversely. We consider this unlikely, because other genes inserted into the B8R locus have either increased (Symons *et al.*, 2002) or decreased (Bartlett *et al.*, 2004) virulence, depending on the nature of the expressed protein. Overall, these data suggest that a phenotype resulting from expression of an immunomodulator by VACV is seen more easily if the parental virus is virulent.

In the i.n. infection model, the attenuated phenotype of the virulent VACV strain expressing 7L was associated with reduced viral titres in the lung and increased numbers of cells recruited to the lungs early after infection. Several studies have demonstrated an increase in chemokine expression during mouse viral infections (Haeberle *et al.*, 2001; Reading *et al.*, 2003) and although the identity of cells producing these chemokines during VACV infection has not been determined, it is likely that chemokines are produced by resident lung cells early after infection and cells recruited to the infected area later on. The extra cells recruited to the lungs by 7L expression were studied, but there was no striking increase in any particular cell type.

In the i.d. model, 7L expression induced a clear reduction in the maximum lesion size and also enhanced redness of the lesion early (days 3–5) after infection. Evidence supporting a role for the CCR8–CCL1 interaction in skin homeostasis and cutaneous immune response has been reported in humans (Schaerli *et al.*, 2004; Gombert *et al.*, 2005). Peripheral T cells (TPs) in healthy skin are CCR8<sup>+</sup> and produce proinflammatory cytokines (tumour necrosis factor alpha and IFN-γ) in response to certain stimuli. These CCR8<sup>+</sup> T<sub>Ps</sub> cells may represent a cutaneous subset of memory T cells able to respond to local antigen challenges and initiate a local inflammatory cascade rapidly without involvement of the LN (Moser & Willimann, 2004).
Possibly, infection with vAB8R-7L may trigger TpK activation to increase inflammation. In addition, CCR8 affects the migration of monocyte-derived DCs to the LN (Qu et al., 2004) and, so, it is possible that the early expression of 7L might enhance the ability of antigen-presenting cells in the dermis to migrate to the LN and induce a rapid antiviral response. The basis of the 7L-mediated attenuation in the i.d. model was addressed by analysis of the type and number of cells recruited to the ears and draining LN, but no significant difference was observed.

In summary, a characterization of the YLDV CKR 7L is provided. We demonstrate that it can be activated in response to murine chemokines in vitro and its expression by VACV can reduce virus virulence in vivo. We hypothesize that the attenuation induced by 7L expression is probably due to infected cells responding differently to chemokines and consequently altering the innate immune response.

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REFERENCES


