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A Soluble Chemokine-Binding Protein from Vaccinia Virus Reduces Virus Virulence and the Inflammatory Response to Infection

Patrick C. Reading,*† Julian A. Symons,‡ and Geoffrey L. Smith,*‡

Many poxviruses express a secreted protein that binds CC chemokines with high affinity and has been called viral CC chemokine inhibitor (vCCI). This protein is unrelated to any known cellular protein, yet can compete with host cellular CC chemokine receptors to modulate host inflammatory and immune responses. Although several strains of vaccinia virus (VV) express a vCCI, the best characterized VV strains Western Reserve and Copenhagen do not. In this study, we have expressed the vCCI from VV strain Lister in a recombinant Western Reserve virus (vΔ8R-R-35K) and characterized its binding properties in vitro and its effect on virulence in vivo relative to wild-type virus (vΔ8R) or a revertant virus (vΔ8R-R) where Lister 35-kDa had been removed. Cells infected with vΔ8R-R-35K secreted a 35-kDa protein that bound the CC chemokine macrophage-inflammatory protein 1α. Expression of vCCI attenuated the virus in a murine intranasal model, characterized by reduced mortality and weight loss, decreased virus replication and spread, and a reduced recruitment of inflammatory cells into the lungs of VV-infected mice. The CC chemokines macrophage-inflammatory protein 1α, eotaxin, and macrophage chemotactic protein 1 were detected in bronchoalveolar lavage fluids from vΔ8R-infected mice; however, bronchoalveolar lavage fluids from vΔ8R-R-35K-infected mice had lower levels of chemokines and a reduced chemotactic activity for murine leukocytes in vitro. These observations suggest that vCCI plays an important role in regulating leukocyte trafficking to the lungs during VV infection by binding to CC chemokines and blocking their chemotactic activities. The Journal of Immunology, 2003, 170: 1435–1442.

The early inflammatory response to a pathogen is important in containing the infection and in shaping the adaptive immune response. Chemokines are a superfamily of chemotactic molecules that are important mediators of the inflammatory response to pathogens (reviewed by Refs. 1 and 2). They modulate movement of leukocytes and up-regulate expression of leukocyte adhesion molecules, and also play important roles in angiogenesis and hemopoiesis. Furthermore, chemokines modify innate and adaptive host responses by enhancing Ag-specific T cell activation and cytokine production (3, 4) and Ag presentation by dendritic cells (5).

Chemokines are small (8–13-kDa) heparin-binding proteins that are classified into C, CC, CXC, or CX3C subfamilies (where X represents any amino acid). The biological activities of chemokines are mediated through interactions with seven-transmembrane receptors expressed by different subsets of target cells, and thus the cellular distribution of different receptors will determine the leukocyte subset that predominates in the inflammatory response. In general, CC chemokines attract macrophages and T cells populations and CXC chemokines attract neutrophils, while lymphotactin (the only C chemokine) attracts T and NK cells. Soluble fractalkine (the only CX3C chemokine) attracts monocytes, T cell subsets, and NK cells. Over 50 chemokines and 16 chemokine receptors have been described (reviewed by Ref. 6).

Poxviruses are large DNA viruses that replicate in the cell cytoplasm and encode many enzymes for transcription and DNA replication (7). In addition, poxviruses also express immunomodulatory factors, capable of blocking components of the host immune system and affecting virus virulence. Such factors interfere with complement and IFN or inhibit apoptosis (reviewed by Ref. 8). In addition, vaccinia virus (VV)† and other poxviruses express secreted binding proteins for IL-1β, IL-18, TNF, IFN-αβ, IFN-γ, and CC chemokines (9).

Chemokines play a critical role in the host response to viral infections (10–12). Not surprisingly, several viruses have evolved strategies to interfere with chemokine function. Many poxviruses express a 35-40-kDa secreted protein that binds to human and rodent CC chemokines with high affinity and competitively inhibits their interaction with cellular receptors (13–15). These proteins, termed T1 for leporipoxviruses or 35-kDa for orthopoxviruses, belong to a related family of proteins known as viral CC chemokine inhibitors (vCCIs) and bear no resemblance to cellular chemokine receptors, nor to other mammalian or eukaryotic proteins. vCCI proteins are encoded by some, but not all, VV strains, cowpox virus, camelpox virus, leporipoxviruses Shope fibroma virus and myxoma virus and by variola viruses (e.g., protein G5R in India 1967) (13–16). The myxoma virus T1 protein (M-T1), but not the related VV 35-kDa (13), also binds to the cell surface via glycosaminoglycans (17).

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The crystal structure of the cowpox virus p35 CC chemokine-binding protein has been determined (18) as well as the specific residues of human macrophage chemotactic protein 1 (MCP-1) that contribute to the interaction of this chemokine with VV 35-kDa (19); interestingly, these residues are conserved among many CC chemokines, and therefore, this provides a structural basis for the ability of VV 35-kDa to recognize many CC chemokines promiscuously. A recent study examining the ability of >80 chemokines to bind vCCI demonstrated high affinity binding to most, but not all, CC chemokines tested and no evidence of binding to CXC, C, or CXC chemokines (20).

In a previous study, several strains of VV and other orthopoxvirus (cowpox and camelpox) were shown to express a secreted 35-kDa vCCI; however, the best-characterized VV strains Western Reserve (WR) and Copenhagen did not (13). In this study, we have constructed a recombinant of VV strain WR expressing the 35-kDa vCCI from VV strain Lister, and examined the influence of vCCI on virulence in a murine intranasal model of infection. The parental virus used was a WR mutant lacking the B8R gene (vB8R) (21). This virus was selected because loss of the B8R gene, which encodes a secreted IFN-γR with low affinity for mouse IFN-γ (21–23), does not affect virus virulence in murine models of infection (21, 24). Expression of vCCI (vΔB8R-35K) led to a marked attenuation compared with wild-type virus (v.WR) (21, 24). Expression of vCCI (vΔB8R-35K) marked animals with reduced mortality, weight loss, and signs of illness, as well as lower levels of virus replication and spread. The attenuated phenotype correlated with a reduction in the cellular inflammatory response in the lungs of mice infected with the vΔB8R-35K virus.

Materials and Methods

Reagents

Radioiodinated recombinant human macrophage-inhibitory protein 1 α (MIP-1α; 2000 Ci/mmol) was obtained from Amersham (Little Chalfont, U.K.). Rabbit antiserum to the purified VV Lister 35-kDa protein was provided by A. Patel (Institute of Virology, Glasgow, U.K.). Goat anti-rabbit IgG conjugated to HRP was obtained from Sigma-Aldrich (Poole, U.K.).

Cell culture

Monkey BS-C-1 and CV-1 cells, human HeLa, D980R, and TK −143 cells, rabbit RK13, and murine L929 and MH-5 cells were grown in DMEM supplemented with 10% FBS (DMEM10).

Viruses

VV strain WR and recombinants thereof were grown in BS-C-1 cells. Construction of a VV WR virus lacking 96% of gene B8R (vΔB8R) was described previously (21). Recombinant VVs and VV Lister strain were grown, titrated, and purified as described previously (25).

Construction of recombinant viruses

A virus expressing the VV Lister 35K gene encoding vCCI (13, 26) from the B8R locus was constructed using transient dominant selection (27). A plasmid, termed pΔB8R, containing 319 and 302 nt of the 5′/3′ flanking sequences, respectively, of gene B8R was described (21). The VV strain Lister 35K gene (26) together with its promoter region containing both early and late transcription start sites (−189 to +820; where n = 1 is the A of the initiation codon) was amplified by PCR with primers 5′-TATCCGGGTTAGTGGCATATACT-3′ (35KRTF1) that contains a SmaI site (underlined) and 5′-CGTTAATATTTACCACTATTCCTCCCC-3′ (35KRB) and plasmid pG620 (26) as template. The PCR product was cloned into pCRScript (Stratagene, La Jolla, CA) generating pCRScript/35K. The 35K gene was excised from this plasmid by digestion with SmaI and cloned into pΔB8R to generate pΔB8R-35K. The orientation of the insert was selected such that the 35K gene was transcribed toward the right end of genome. The DNA sequence was determined and shown to be correct.

Plasmid pΔB8R-35K was transfected into VV (vΔB8R)-infected cells and mycophenolic acid-resistant viruses were isolated as described (27).

These were grown on hypoxanthine guanine phosphoribosyltransferase-negative D980R cells in the presence of 6-thioguanine (28), and plaque isolates corresponding to original virus (vΔB8R) or 35K recombinant (vΔB8R-35K) were identified by PCR using oligonucleotides that span the B8R gene locus. A revertant virus (vBB8R-R) was constructed by transfecting plasmid pΔB8R into vΔB8R-35K-infected cells. Mycophenolic acid-resistant intermediate viruses were isolated as above and resolved into those containing the 35K gene (vΔB8R-35K) and revertant viruses (vBB8R-R) on D980R cells in the presence of 6-thioguanine as described.

Immunoblot

TK −143B cells were infected with VVs at 10 PFU/cell for 24 h (unless otherwise stated) and the supernatants were resolved as described previously (27). All samples were resolved by SDS-PAGE on 12.5% gels. After electrophoretic transfer of proteins to nitrocellulose membranes, the blots were incubated sequentially with rabbit serum to purified Lister 35-kDa protein diluted 1/2500 (a gift from A. Patel (Institute of Virology)), a goat anti-rabbit peroxidase conjugate (diluted 1/1000), and ECL reagents (Amersham).

Ligand binding

Cross-linking experiments with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 125I-labeled recombinant human MIP-1α (2000 Ci/mmol, Amersham) were performed in a volume of 25 µl as described previously (13). Samples were analyzed by SDS-PAGE and autoradiography.

Assays of viral virulence

Six- to 8-week-old female BALB/c mice were anesthetized and infected intranasally with 20 µl of diluted virus in PBS. Infectious doses ranged from 103 to 108 PFU. Each day, mice were weighed individually and monitored for signs of illness, and those having lost >30% of body weight were sacrificed. To determine virus titer in organs, mice were sacrificed and pooled with 10 days postinfection (p.i.), and their brains and lungs were removed, dounced homogenized in 1 ml of PBS, frozen and thawed three times, and sonicated. Virus infectivity was determined by plaque assay on BS-C-1 cells. Virus titers were verified before and following each experiment to ensure the accuracy of the doses administered.

Bronchoalveolar lavage (BAL) and differential analysis of recovered cells

For collection of BAL cells, mice were sacrificed and the lungs were flushed five times with a 1-ml volume of PBS containing 10 U/ml heparin through a blunted 23-gauge needle inserted into the trachea. The five lavage samples were pooled and the cells were treated with Tris-NH4Cl (0.14 M NH4Cl in 17 mM Tris, adjusted to pH 7.2) to lyse erythrocytes, washed twice, and resuspended in cold RPMI 1640 medium supplemented with 10% FBS. Aliquots of −5 × 105 BAL cells were cytostecondensed on microscope slides for 10 min at 800 rpm in a Shandon (Pittsburgh, PA) cytocentrifuge. The slides were dried in air, and the cells were fixed and stained with H&E, and blinded differential counts were performed. Wells were viewed under ×400 magnification, and cells in at least four random fields were assessed (>200 cells/field).

Evaluation of chemokine protein levels by ELISA

Levels of chemokines in mouse BAL fluids were measured by commercial ELISA kits for MIP-1α (R&D Systems, Abingdon, U.K.) and MCP-1 (BD PharMingen, San Diego, CA), and eotaxin levels were determined by sandwich ELISA using matched Ab pairs from R&D Systems according to the manufacturer’s instructions. The sensitivities of the assay for MIP-1α, MCP-1, and eotaxin were 5, 15, and 10 pg/ml, respectively. Reagents for MCP-1 and eotaxin ELISAs were kindly supplied by C. Lloyd and L. Murray (Biomedical Sciences Division, Faculty of Medicine, Imperial College). BAL samples and culture supernatants were centrifuged at 1000 rpm or 10 min before the concentration of chemokines was determined.

Chemotaxis assays

The chemotactic activity of BAL fluid was evaluated using 10-mm tissue culture inserts (Life Technologies, Grand Island, NY) placed in 24-well plates. Mediastinal lymph node (MLNs) were collected from mice 10 days after intranasal infection with 105 PFU of vΔB8R, and single-cell suspensions were prepared by sieving through a 100-μm nylon mesh in 5 ml of RPMI 1640 with 1 mg/ml BSA. Cell viability was assessed by trypan blue exclusion, and cells were adjusted to 1.5 × 106 cells/ml. A 0.5-ml aliquot of PBS alone or BAL fluid was placed in the lower compartment, and 0.5 ml of MLN cell suspension was placed in
the upper chamber, separated by a polycarbonate filter (8-μm pore size). After incubation at 37°C for 2 h, the filter was washed, fixed, and stained with 4′,6′-diamidino-2-phenylindole. The number of migrating cells in five high-powered (×400) fields was counted using a fluorescence microscope.

**Results**

**Construction of rVV strains**

Several strains of VV express a soluble vCCI; however, the best characterized VV strains WR and Copenhagen do not (13). The VV Lister “35-kDa gene” encodes vCCI (13). A VV mutant lacking this gene (ListerΔ35K) was reported (26); however, this virus was unsuitable for examining the role of vCCI in virus virulence because of the mild virulence of VV Lister in mice and the lack of a revertant control. Therefore, the role of vCCI in vivo was investigated by construction of a rVV strain WR that expressed Lister vCCI. The parental virus selected was a mutant of WR that lacked the B8R gene (21). B8R encodes an IFN-γR that has a low affinity for mouse IFN-γ, and does not affect virulence in murine models of infection (21, 24). However, the deletion mutant is likely to be attenuated in man, because the B8R protein binds and inhibits human IFN-γ (21–23). A recombinant virus expressing the Lister 35-kDa gene (vΔB8R-R) and a control virus, in which the 35-kDa gene was removed from vΔB8R-R (vΔB8R-R), were constructed (see Materials and Methods). Analysis of the genomes of parental (vΔB8R), vΔB8R-35K, or vΔB8R-R viruses by PCR confirmed that the entire 35-kDa gene was present in vΔB8R-35K and that the genes flanking the B8R locus were unchanged in each of the viruses (data not shown).

To investigate whether the addition of the vCCI affected virus growth in vitro, BS-C-1 cells were infected at 0.01 PFU/cell, and the combined infectivity present in the cells and supernatant was determined over a 48-h period. No differences were observed between the viruses in the rate of increase in virus titer or the final titer attained (data not shown). Furthermore, the plaque morphology formed on BS-C-1 cells by each of the virus isolates was indistinguishable (data not shown), indicating that expression of the vCCI does not affect virus replication in vitro.

**Expression of vCCI by rVV strains**

Expression of a soluble vCCI by VV strains WR and Lister, and the recombinant WR viruses vΔB8R, vΔB8R-35K, or vΔB8R-R, was examined by immunoblot using supernatants from VV-infected cells and an antiserum raised against the 35-kDa protein from VV Lister. A protein of ~35 kDa was detected in the supernatant of cells infected with VV Lister that was absent from WR- and mock-infected samples (Fig. 1A). A similar protein was also detected in supernatants from cells infected with vΔB8R-35K, but not vΔB8R or vΔB8R-R, consistent with expression of the Lister 35-kDa protein by vΔB8R-35K.

Next, we determined the expression of the 35-kDa protein over time in cells infected with VV Lister or vΔB8R-35K. A 35-kDa protein was detected in the supernatant of cells infected with Lister and vΔB8R-35K by 4 h p.i., and this increased up to 18 h p.i. (Fig. 1B). The protein was also detected in the presence of cytosine arabinoside, an inhibitor of DNA replication and therefore late gene expression, demonstrating that the protein is expressed both early and late in infection with VV Lister or vΔB8R-35K.

To confirm that the 35-kDa protein secreted from vΔB8R-35K-infected cells was a CC chemokine-binding protein, we performed binding assays with 125I-labeled MIP-1α and supernatants from mock-infected or VV-infected cells, followed by chemical cross-linking with EDC and analysis by SDS-PAGE and autoradiography. The addition of MIP-1α to culture supernatants resulted in a complex of ~40 kDa from Lister- and vΔB8R-35K-infected cells (Fig. 1C). Supernatants from uninfected cells, or cells infected with WR, vΔB8R, or vΔB8R-R, did not contain any detectable complexes. Taken together, these results indicate that cells infected with vΔB8R-35K secrete a 35-kDa protein that binds CC chemokines.

**Expression of vCCI attenuates VV strain WR in mice**

Although the addition of the Lister 35-kDa had no effect on replication of vΔB8R-35K in cell culture, it was likely to affect virus virulence in vivo, and this was examined in a murine intranasal model. Groups of five mice were infected with 10³, 10², 10¹, or 10⁰ PFU of vΔB8R, vΔB8R-35K, or vΔB8R-R, and then observed for 14 days. It is clear that expression of the Lister 35-kDa protein

![FIGURE 1. Expression of Lister vCCI by vΔB8R-35K.](http://www.jimmunol.org/...)

- A 35-kDa protein is expressed by VV Lister and by vΔB8R-35K.
- TK 143B cells were mock-infected or infected with the indicated VVs, and 24 h later, proteins in the supernatants were separated by SDS-PAGE and transferred to nitrocellulose. Blots were incubated with an antiserum against Lister 35-kDa protein, and bound Ab was detected as described in Materials and Methods.
- The Lister 35-kDa protein is made early and late during infection.
- Cross-linking of 125I-labeled MIP-1α with EDC to supernatants from mock-infected cultures, or cultures infected with the indicated VVs. The amount of supernatant used was equivalent to 2×10⁴ cells. An autoradiograph of the SDS-PAGE analysis, with molecular masses in kilodaltons, is shown, and an arrow indicates the ligand-receptor complexes.
from vΔB8R diminished virus virulence (Table I); this was seen best at an inoculum dose of 10⁵ PFU where all control animals were sacrificed at humane endpoints, while all animals infected with vΔB8R-35K survived.

Virus virulence was investigated further in a second experiment where mice were weighed and assessed for general signs of illness daily. The results are shown in Fig. 2 and confirm that vΔB8R-35K is attenuated in the murine intranasal model. At 10⁷ PFU, the mean weight loss following infection with vΔB8R-35K was less severe than with control viruses (Fig. 2A), and this was reflected in milder signs of illness (Fig. 2B). At 10⁵ PFU, mice infected with vΔB8R and vΔB8R-R lost weight rapidly and all animals were sacrificed by 8 days p.i. (Fig. 2C), while vΔB8R-35K-infected animals showed a milder weight loss and all animals survived the infection. The illness observed after inoculation of mice with 10⁵ PFU of vΔB8R or vΔB8R-R was severe, characterized by symptoms of pneumonia at the time of sacrifice; however, animals infected with the same dose of vΔB8R-35K displayed only mild signs of disease (Fig. 2D).

### Table 1. Mortalities at 14 days p.i. with viruses vΔB8R, vΔB8R-35K, and vΔB8R-R

<table>
<thead>
<tr>
<th>Dose of Virus (PFU)</th>
<th>vΔB8R</th>
<th>vΔB8R-35K</th>
<th>vΔB8R-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>10³</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
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<td>10⁴</td>
<td>0/5</td>
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<tr>
<td>10⁵</td>
<td>5/5</td>
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<td>5/5</td>
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<tr>
<td>10⁶</td>
<td>Not done</td>
<td>5/5</td>
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</table>

*Animals were infected as described in Materials and Methods, and those animals that were sacrificed at humane end points within the 14 days p.i. are shown.*

In the murine intranasal model, inoculum doses of >10⁴ PFU are accompanied by extensive respiratory infection, virus dissemination, and death (29, 30). The replication of VV in the lungs and brains of mice was determined 5, 7, and 10 days p.i. with 10⁴ PFU of vΔB8R, vΔB8R-35K, or vΔB8R-R (Fig. 3). Less virus could be recovered from the lungs of vΔB8R-35K-infected mice at days 5 and 7 p.i.; however, this difference was most striking at day 10 when virus titers were reduced 10- to 100-fold compared with animals infected with control viruses. Two of the five animals infected with vΔB8R-35K had cleared the lung infection by day 10. Dissemination of virus within vΔB8R-35K-infected animals was even more restricted, and by day 7, no virus could be recovered from the brain, whereas the majority (9 of 10) of mice infected with vΔB8R or vΔB8R-R yielded virus. Low levels of virus could still be detected in the brain of some (6 of 10) vΔB8R- or vΔB8R-R-infected mice 10 days p.i.

The dissemination of VV into the brain and/or associated meningeal tissues following intranasal infection is probably due to hematogenous spread after initial replication in the lung parenchyma. Consistent with this, while high levels of infectious virus were recovered from lungs of mice 1 and 3 days p.i. with 10⁴ PFU of vΔB8R, we could not detect virus in brain homogenates until day 3 p.i. (data not shown). To investigate bloodborne virus, mice were infected with 10⁴ PFU of vΔB8R, vΔB8R-35K, or vΔB8R-R, and viremia was evaluated on days 3 and 5 p.i. On day 3, virus was recovered from the blood of vΔB8R-, vΔB8R-35K-, and vΔB8R-R-infected mice (2.1 ± 0.5, 1.6 ± 0.3, and 1.9 ± 0.5 log₁₀ PFU/ml of blood from 3 of 5, 2 of 5, and 3 of 5 infected animals, respectively) but only from vΔB8R- and vΔB8R-R-infected mice at day 5 p.i. (2.2 ± 0.4 and 2.0 ± 0.6 log₁₀ PFU/ml of blood from 3/5 and
4/5 infected animals, respectively). No virus could be recovered from blood of animals infected with 10^4 PFU of vΔB8R-35K at day 5 (<1.5 PFU/ml of blood, 0 of 5 infected animals), suggesting less effective viremic spread by this virus.

**Cellular inflammatory response to VV in the respiratory tract**

Intranasal infection of mice with WR leads to an extensive respiratory infection characterized by high levels of virus replication and infiltration of inflammatory cells into the lung. As chemokines are involved in the activation and recruitment of inflammatory cells to sites of virus infection, we investigated the cellular inflammatory response in the lungs of mice infected with 10^4 PFU of vΔB8R, vΔB8R-R, vΔB8R-35K, or vΔB8R-R.

First, the mean lung weights from VV-infected animals were compared. vΔB8R- and vΔB8R-R-infected mice had lung weights significantly greater than those from vΔB8R-35K-infected animals at 7 and 10 days p.i. (Fig. 4A), consistent with the induction of edema and inflammation in mice lacking the vCCI. There was a marked consolidation of the lungs in vΔB8R- and vΔB8R-R-infected mice by day 7 p.i., suggesting pneumonia.

Second, cells infiltrating the alveolar and lung spaces were recovered by lavage, counted, and stained to examine the nature of the inflammatory infiltrate. Cell numbers in BAL increased 5- to 8-fold following infection with vΔB8R and vΔB8R-R, and peak numbers were seen 10 days p.i. (Fig. 4B). Cell numbers remained elevated at day 15 despite the absence of detectable virus in the lungs at this time (data not shown). Fewer cells were recovered from the lungs of mice infected with vΔB8R-35K at days 5, 10, and 15, indicating that expression of vCCI by this virus inhibited migration of leukocytes into the VV-infected lung.

Differential counts indicated that the inflammatory cells present in BAL from VV-infected mice were predominantly macrophages/monocytes and lymphocytes. Less than 5% of total BAL cells were granulocytes at any time tested (data not shown). Fewer macrophages (Fig. 4C) and lymphocytes (Fig. 4D) were recovered in BAL fluids from vΔB8R-35K-infected mice, consistent with a role for the Lister 35-kDa protein in inhibiting chemokine-mediated recruitment of these cells in vivo.

**Chemokine levels in BAL fluid and culture supernatants during VV infection**

Although many orthopoxviruses encode an inhibitor of CC chemokines, reports examining the ability of these viruses to induce CC chemokines in vitro or in vivo are few (31). Therefore, we examined levels of the CC chemokines MIP-1α, eotaxin, and MCP-1 in BAL fluids from VV-infected mice. Although chemokines will be diluted considerably during lavage, we were still able to detect all three at the protein level during VV infection (Fig. 5). CC chemokine levels peaked 7 days after vΔB8R-35K infection and had fallen away markedly by day 10. However, after infection with vΔB8R or vΔB8R-R, levels of MIP-1α, MCP-1, and eotaxin...
A41LHis produced in the baculovirus system, and their ability to induce infected mice, or from mice 7 days p.i. with 10⁴ PFU of vΔB8R, vΔB8R-35K, or vΔB8R-R, and their ability to induce leukocyte migration was determined in a chemotaxis assay. Single-cell suspensions were prepared from MLNs of mice infected 10 days previously with 10⁵ PFU of vΔB8R. The mean number of migrating MLN cells per field ± SEM under high power (n = 5) is shown. BAL supernatants from mice 7 days p.i. with 10⁵ PFU of vΔB8R were preincubated with 10 nM purified 35K.His or A41L.His produced in the baculovirus system, and their ability to induce migration of MLN cells was determined. The mean number of migrating MLN cells per field ± SEM under high power (n = 5) is shown.

were elevated by day 7 p.i., but had increased further by day 10, consistent with the heightened influx of inflammatory cells seen in the lungs of these animals at this time (Fig. 4).

Chemotactic activity in BAL fluid from VV-infected mice

The results shown in Fig. 5 demonstrated that VV infection upregulates production of a number of CC chemokines in BAL fluids in vivo. We next compared the chemotactic activity of BAL fluid from mice infected with vΔB8R, vΔB8R-35K, or vΔB8R-R for cells from the MLNs of VV-infected mice (Fig. 6A). Using a microchemotaxis assay, we found BAL fluid taken 7 days after intranasal infection with vΔB8R or vΔB8R-R was able to induce the migration of MLN cells more effectively than BAL from vΔB8R-35K-infected mice, despite enhanced levels of CC chemokines present in day 7 vΔB8R-35K BAL (Fig. 6). This finding is consistent with expression of a vCCI able to bind a broad spectrum of CC chemokines by the vΔB8R-35K virus. BAL fluid from mock-infected animals displayed negligible chemotactic activity. In a second experiment, recombinant 35-kDa protein containing a C-terminal six-histidine tag (35K-His) was produced from recombinant baculoviruses and purified by metal chelate affinity chromatography as described (13). Another VV protein, A41L, was also expressed in the baculovirus system with a six-histidine tag (32), and was purified and used as a control (A41L-His). Preincubation of 35K-His, but not A41L-His, with BAL fluids from day 7 vΔB8R-R-infected mice inhibited the migration of MLN cells (Fig. 6B).

Discussion

Recruitment of leukocytes to sites of infection is crucial to the inflammatory clearance of viruses and other pathogens. CC chemokines play a critical role by recruiting and inducing a range of biological effects upon subpopulations of leukocytes such as mononuclear cells and T and B lymphocytes. In this study, we have examined the biological significance of the vCCI in VV infection by comparing the virulence of a parental virus (VV WR lacking gene B8R, vΔB8R), a recombinant WR virus expressing vCCI (vΔB8R-35K), and a revertant virus lacking vCCI (vΔB8R-R) in a murine intranasal model of infection. Expression of VV strain Lister vCCI reduced virus virulence and the host inflammatory response to infection. The CC chemokines MIP-1α, MCP-1, and eotaxin were detected in BAL fluids of mice after infection with vΔB8R, vΔB8R-35K, and vΔB8R-R; however, expression of vCCI blocked the ability of BAL fluid to induce leukocyte chemotaxis in vitro. In vivo, fewer leukocytes were recovered from lungs of mice infected with vΔB8R-35K compared with those of controls.

The reduced virulence of VV WR expressing vCCI is consistent with a report that intranasal infection of mice with a rabies virus (RPV) mutant lacking the gene encoding the 35-kDa vCCI (RPVΔ35) led to a modest enhancement in weight loss and clinical signs of illness (33). At lower doses (10³ PFU), more severe disease symptoms were noted in mice infected with the RPVΔ35 mutant than with the parental virus; however, this effect was less evident at higher doses and a revertant virus was not included. In comparison, the attenuation we observed following the expression of the Lister vCCI from VV vΔB8R is quite striking. Consider the mortality data (Table I) where all mice infected with vΔB8R-35K survived infection with 10³ PFU, while all vΔB8R-R- and vΔB8R-R-infected animals did not. The more pronounced influence of vCCI on VV virulence could be explained in a number of ways. First, expression of vCCI may differ between our rVV vΔB8R-35K and RPV. We have shown the vCCI from vΔB8R-35K is consistent with expression at similar times p.i. and at similar, albeit slightly lower levels, than that from VV strain Lister (Fig. 1, A and B), while vCCI/35-kDa was found to be a major secreted protein in the supernatants from RPV-infected cells (33). The slight difference in expression levels might reflect the diploid nature of the gene in VV Lister and RPV due to its presence within the inverted terminal repeat, but only a single copy in vΔB8R-35K. Second, and more likely, the greater influence of vCCI on VV virulence may be associated with differences in pathogenesis observed between RPV and VV strain WR. The cellular infiltrate induced following intranasal infection with RPV was reported to be composed largely of lymphocytes and neutrophils (34); however, we report neutrophils to be only a minor component of the cellular inflammatory response to VV (<5% of total BAL cells at any time point examined in our study; Fig. 4). CC chemokines are particularly important in modulating recruitment and activation of macrophages and lymphocyte subpopulations rather than neutrophils; thus, inhibition of CC chemokines by vCCI might be expected to exert more dramatic effects on the cellular inflammatory response to VV infection.

Studies of poxvirus pathogenesis have used a number of different experimental animal models and/or inoculation routes to examine the role of vCCIs, but all corroborate our finding that these proteins can modulate the cellular inflammatory response to infection. Rabbits infected intradermally with RPVΔ35 showed no overall differences in disease pathogenesis (33), although histological analysis of RPV-infected rabbit lesions demonstrated that RPVΔ35 enhanced leukocyte trafficking during the early phase of infection (14). Furthermore, deletion of vCCI M-T1 from myxoma virus was reported to heighten the localized cellular inflammation during the early phase of infection (35). It is interesting to note that, for both RPV and myxoma virus infections, the enhanced cellular influx seen in rabbits infected with the vCCI-deficient viruses was not associated with a more effective virus clearance, suggesting that perhaps other poxvirus proteins may interfere with the antiviral activity of the infiltrating cells.

The expression of vCCI from VV strain WR was associated with a reduced cellular infiltrate into the lung, yet the virus titers...
and virulence were markedly reduced in vivo. Although these findings may at first seem paradoxical, a number of explanations can be proposed. First, in addition to mediating virus clearance, the cellular influx characteristic of VV infection of the lung might contribute to pathology. Inflammatory responses in the lung must be tightly controlled, because an excessive accumulation of inflammatory cells could be harmful, and contribute to the clinical symptoms and tissue damage that are typical of virus-induced pneumonia. Animals infected with v\Delta88R-35K displayed little evidence of ruffled fur, decreased activity, or respiratory distress consistent with a role for recruited cells in the development of the pneumonia seen in v\Delta88R- and v\Delta88R-R-infected animals (Fig. 2B). Second, recruitment of inflammatory cells to the lung may aid in virus spread throughout the body. Although in vitro studies failed to demonstrate productive infection of alveolar macrophages (data not shown), it is possible that virus adsorbed to the surface of lymphocytes or macrophages could be carried to sites of secondary replication such as the brain, spleen, and liver. In the absence of an excessive inflammatory response, other immune mediators may also act more effectively against virus-infected cells. Local production of TNF-α and IFN-γ in the lung may be particularly important, given that VV strain WR does not encode a TNFR (36) and that the soluble IFN-γR encoded by VV has a low affinity for (21) and does not neutralize murine IFN-γ (22).

The cellular and molecular mechanisms underlying VV-induced lung pathology are not well understood; however, it is likely that chemokines are involved in modulating the cellular inflammatory response to infection. Previous studies have shown the CXC chemokines known as monokine induced by IFN-γ and cytokine-responsive gene 2 to be induced in multiple organs following VV infection (31), and rVV expressing either of these chemokines was responsive to VV infection, e.g., the induction of an antiviral effect in vivo (12). In this study, we have demonstrated that the CC chemokines MIP-1α, MCP-1, and eotaxin are induced in the lung in response to VV infection, although it is unknown whether these are produced by infected or uninfected cells. MIP-1α, a CCR3-binding chemokine, acts as a potent chemotactrant for monocytes and activated T cells (37, 38), two of the major components of VV-induced inflammation of the lung. Similarly, MCP-1, which binds to the CCR2 receptor is a potent chemotactrant and activator of monocytes (39). MIP-1α plays a central role in mediating inflammatory responses in the lung following infection with influenza, pneumonia virus of mice, and respiratory syncytial virus (10, 40, 41). Despite the fact that both eotaxin and MIP-1α are known chemokine--activating ligands for eosinophils, we were unable to demonstrate eosinophil influx of VV-infected mice, suggesting that additional factors (e.g., IL-5) (42) may be necessary to fully activate the multistep process required to recruit eosinophils into lung tissue. Future studies will be directed toward characterizing the chemokine profile induced in response to VV infection, e.g., the induction of additional CC chemokines (RANTES, MIP-1β, and T cell activation 3) as well as CXC and other chemokines. Moderate to low levels of CXC chemokines, including KC and MIP-2, might be predicted in accordance with the low numbers of neutrophils recovered from the lungs of VV-infected mice. Alternatively, poxviruses such as VV may have evolved additional strategies to counteract the recruitment and activation of neutrophils.

Taken together, the results of our study indicate that expression of a VCCI from VV strain WR, which does not normally express such an activity, inhibited the cellular inflammatory response induced to the virus in vivo. These findings are consistent with previous reports demonstrating a role for VCCIs in modulating the cellular inflammatory response to RPV and myxoma virus; however, in contrast to these studies, we also present evidence that this protein can play a major role in viral virulence. The broad neutralizing activity of vCCI for CC chemokines could prove useful in the treatment of inflammatory conditions. To this end, administration of vCCI to the respiratory tract was reported to be highly effective in blocking the inflammatory and airway physiological consequences of allergen-induced asthma in a murine model, without altering systemic Ag-specific immunity (43).

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References