Severe Murine Lung Immunopathology Elicited by the Pathogenic Equine Herpesvirus 1 Strain RacL11 Correlates with Early Production of Macrophage Inflammatory Proteins 1α, 1β, and 2 and Tumor Necrosis Factor Alpha

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The CBA mouse model was used to investigate the immunopathology induced in the lung by the pathogenic equine herpesvirus 1 (EHV-1) strain RacL11 in comparison to infection with the attenuated vaccine candidate strain KyA. Intranasal infection with KyA resulted in almost no inflammatory infiltration in the lung. In contrast, infection with the pathogenic RacL11 strain induced a severe alveolar and interstitial inflammation, consisting primarily of lymphocytes, macrophages, and neutrophils. Infection with either EHV-1 strain resulted in the accumulation of similar numbers and ratios of CD4 and CD8 T lymphocytes in the lung and bronchoalveolar lavage (BAL) fluid. Further analysis of these T-cell populations revealed identical EHV-1-specific cytotoxic T-lymphocyte responses. RNase protection analysis of RNA isolated from the BAL fluid of RacL11-infected mice on day 3 postinfection revealed much higher levels of RNA specific for macrophage inflammatory protein 1α (MIP-1α), MIP-1β, and MIP-2 than were observed for KyA-infected mice. Furthermore, significantly higher levels of transcripts specific for tumor necrosis factor alpha were induced on day 3 postinfection with RacL11 compared with KyA. These findings suggest that the early production of proinflammatory beta chemokines plays a major role in the severe, most often lethal, respiratory inflammatory response induced by the pathogenic EHV-1 strain RacL11.

Naturally occurring mucosal infection of the horse with equine herpesvirus 1 (EHV-1) typically results in respiratory distress, abortogenic disease, and, albeit rarely, severe neurological sequelae (5, 13, 24, 30, 31, 32). By far the most devastating outcome of EHV-1 infection of the horse is the induction of abortion in pregnant mares, which has a severe economic impact on the equine industry. EHV-1-induced abortion in pregnant mares requires a sequential infection of the respiratory epithelium, followed by infection of mononuclear cells and T cells, resulting in a cell-associated viremia and subsequent infection of endothelial cells within the endometrial vasculature (6, 10, 40). Currently, there are no available EHV-1 vaccines that elicit long-term immunity and protection in the horse (11, 12, 22).

The mouse model of EHV-1 infection was originally established in various mouse strains (2, 7). We recently adapted this model to CBA (H-2k) mice to allow characterization of the primary and memory EHV-1-specific cytotoxic T-lymphocyte (CTL) responses to the attenuated vaccine candidate EHV-1 strain KyA (15, 28, 41). In these studies it was reported that KyA afforded protection in the mouse and the horse against subsequent infection with the highly pathogenic EHV-1. It was observed that KyA infection of CBA mice resulted in no clinical signs of infection. In contrast, infection of CBA mice with the pathogenic RacL11 strain resulted in severe weight loss, ruffled fur, huddling behavior, and eventually death between days 6 and 8 postinfection (41, 48).

Those observations strongly suggested a fundamental difference between these two EHV-1 strains in growth potential in vivo and/or in the host-virus interaction. Measurement of virus levels in the lungs on days 2 through 6 postinfection indicated that the pathogenic RacL11 strain was cleared from the lung tissue with kinetics identical to those observed following infection with the attenuated KyA strain. Infection with either EHV-1 strain resulted in peak viral titers on day 2 postinfection, and infectious virus was completely eliminated from the lung tissue by day 6 (40). Since these mice succumbed to RacL11 infection on days 6 to 8 postinfection, it was speculated that death was likely the result of the host interaction with EHV-1 RacL11, and not the result of inability to clear this EHV-1 strain from the lung. A clear understanding of the immune mechanisms that constitute a protective “appropriate” response versus a potentially damaging “inappropriate” response and the identification of viral components responsible for eliciting those responses are imperative for the development of an immunoprophylactic vaccine.

In the present study, we examined the acquired immune response in the infected lung following infection with EHV-1 KyA or RacL11 and the subsequent immunopathology as a result of that response. We found that intranasal (i.n.) infection with the pathogenic RacL11 strain results in a severe inflammatory infiltration involving the majority of the lung tissue, a finding not observed following KyA infection. Lung sections taken from RacL11-infected mice revealed a massive cellular consolidation of the lung, consisting primarily of lymphocytes, macrophages, and neutrophils. Lung sections from...
KyA-infected mice were almost completely clear of inflammatory infiltration, closely resembling sections taken from mock-infected mice. These results suggest that, while the immune response elicited by KyA is protective, the response to RacL11 is damaging and results in the death of the animal. Immune mechanisms potentially playing a role in this inappropriate response and leading to severe immunopathology of the lung are characterized and discussed.

MATERIALS AND METHODS

**Virus and cell culture.** EHV-1 KyA and RacL11 viral stocks used for i.n. infection of mice were propagated on L2 mouse fibroblast monolayers. Titers of both virus strains were determined by a differential count of at least 200 stained cells per sample. In all experiments, mouse groups consisted of at least five mice each. CTL assays and RNase protection analysis were performed using pooled cells isolated from groups of five mice. All experiments were approved by the University Animal Care Committee. All mice were housed in filter-topped cages. This facility is certified by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animal procedures were performed by the University Animal Care Committee. All mice were rested a minimum of 1 week prior to use. In all experiments, mouse groups consisted of at least five mice each. CTL assays and RNase protection analysis were performed using pooled cells isolated from groups of five mice. All experiments were performed a minimum of three times.

**Histopathology.** Representative lungs from groups of infected (i.n., with $2 \times 10^6$ PFU of KyA or RacL11) mice were removed at 5 days postinfection, fixed in 10% buffered formalin for 24 h, embedded in paraffin, and sectioned at 5 μm. Sections were stained with hematoxylin and eosin.

**Identification and quantitation of cells isolated from the BAL fluid.** Mice were infected i.n. with $2 \times 10^6$ PFU of KyA or RacL11, and the bronchoalveolar lavage (BAL) fluid was recovered on day 5 postinfection as follows. Mice were sacrificed by halothane inhalation, and the pleural cavity was exposed. The trachea was cut just below the larynx, and a smooth-tipped 20-gauge needle was inserted into the trachea. The needle was secured in place by tying with waxed dental floss. BAL fluid was obtained by using a 1-ml syringe to infuse 1-ml aliquots of phosphate-buffered saline (PBS) in and out of the lungs five times for a total of 5 ml. Cells isolated from the BAL fluid were then air dried onto a glass slide, fixed for 5 min in methanol, stained for 20 min with Giemsa stain, and rinsed with double-distilled water. Cell types were identified by standard morphological evaluation under light microscopy, and percentages of each cell type were determined by a differential count of at least 200 stained cells per sample. The data are presented as mean percentages over a range of five separate experiments.

**Infection and assessment of CTL activity.** CBA mice were anesthetized with halothane (Sigma Chemical Co., St. Louis, Mo.) and inoculated i.n. with $2 \times 10^6$ PFU of EHV-1 KyA or RacL11 in an inoculum volume of 50 μl. To assess primary CTL activity in the lung at 5 days postinfection, lymphocytes were isolated from the lungs as follows. The lungs were removed in the absence of the heart and placed on a gelatinized slide, with the tissue being maintained on the gelatinized slide, pressed through a 60-gauge screen and digestion with DNase-collagenase as described above. RNA was isolated from the resulting cells by using the TRIZOL reagent (Life Technologies, Grand Island, N.Y.) according to the manufacturer's protocol. Protected RNA samples and appropriate standards were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 5% acrylamide gels, which were then blotted onto filter paper, dried, and exposed to film. The resulting film was scanned using the UV Transilluminator 2000 (Bio-Rad, Hercules, Calif.), and bands were quantitated by using the Quantity One program (Bio-Rad) according to the manufacturer's protocol.

**RESULTS**

**Flow cytometric analysis of T cells isolated from the lung.** The striking differences between the outcomes of infection with KyA versus RacL11 strongly suggested that a fundamental difference exists between the ways these two strains grow in vivo and/or between the immune responses elicited to them. The former was ruled out by our previous demonstration that infectious RacL11 and KyA are completely cleared from the lung tissue by day 6 postinfection with identical kinetics (41). These results and the observation that the mice exhibited labored breathing before succumbing to infection suggested that the fatality observed following RacL11 infection might be the result of severe immunopathology in the lung. Flow cytometric analysis revealed that CD4 and CD8 T cells isolated from the entire lung were present in almost identical CD4/CD8 ratios of approximately 2:1 at 5 days following infection with either KyA or pathogenic RacL11 (Fig. 1). Interestingly, in the BAL fluid on day 5 postinfection, CD8 T cells were the predominant lymphocyte population, and a CD4/CD8 ratio of approximately 0.6:1 was observed in both RacL11- and KyA-infected mice (Fig. 1). Although T cells were present in the lungs of KyA- and RacL11-infected mice in comparable numbers and ratios, the total number of cells isolated from RacL11-infected lungs, most likely infiltrating monocytes and granulocytes, was generally 5- to 10-fold greater (data not shown). These results suggested a much more vigorous inflammatory infiltration into the lungs prior to infection with RacL11.

**Assessment of inflammatory infiltration in the infected lung.** Macroscopic observation of KyA- and RacL11-infected lungs revealed a much more extensive inflammatory response following RacL11 infection (data not shown). The consolidation involved more than 90% of the total lung tissue compared to that observed following infection with KyA. Although inflammation was present in KyA-infected lungs compared to uninfected lungs, it encompassed only approximately 10 to 20% of the total lung. Histological analysis of infected lungs confirmed these observations. Figure 2A and B show the typical appearance of uninfected CBA lung tissue. On day 5 postinfection, KyA-infected lungs exhibited mild perivascular and peribronchial inflammation, consisting mostly of lymphocytes, with little or no infiltration into the alveolar spaces (Fig. 2C and D). The inflammatory cells infiltrating the RacL11-infected lung also appeared to be primarily lymphocytes (Fig. 2E and F). However, the lesion present in the RacL11-infected lung appeared much more severe, exhibiting diffuse alveolar damage (DAD) with alveolar edema and diffuse interstitial infiltration (Fig. 2E). Further, leakage of protein-rich fluid from the alveolar capillaries into the alveoli resulted in the formation of hyaline membranes lining the alveolar walls (Fig. 2F). Morphological examination and quantitation by light microscopy of cells isolated from the BAL fluid revealed that the
BAL fluid of RacL11-infected mice consisted of lymphocytes, macrophages, and neutrophils (Table 1). In contrast, the infiltrating cells isolated from the BAL fluid of KyA-infected mice consisted of lymphocytes and macrophages; no neutrophils were detected (Table 1). We have observed previously that consolidation within the RacL11-infected lung was most severe when viral titers were at their lowest (days 5 and 6 postinfection). Taken together, these results suggested that fatality following RacL11 infection was likely due to severe DAD and was independent of levels of infectious virus (41).

FIG. 1. Flow cytometric analysis of lymphocytes isolated from EHV-1-infected lungs on day 5 postinfection. CBA mice were infected i.n. with 2 × 10^6 PFU of EHV-1 KyA or RacL11. At 5 days postinfection, the lungs were removed, and lymphocytes were isolated by collagenase-DNase digestion as described in Materials and Methods. BAL fluid was obtained at day 5 postinfection as described in Materials and Methods. The resulting lymphocytes were then double stained with FITC-CD3ε and PE-CD4 or PE-CD8 and were analyzed by flow cytometric analysis.

FIG. 2. Histological sections of infected lungs at 5 days postinfection. Lungs were infused with 10% buffered formalin and removed 5 days following mock infection (A and B) or i.n. infection with 2 × 10^6 PFU of KyA (C and D) or RacL11 (E and F). Paraffin sections were cut and stained with hematoxylin and eosin.
Analysis of cytolytic activity of lymphocytes isolated from infected lungs. The severe inflammatory response in the lungs of RacL11-infected mice, present in the absence of infectious RacL11, suggested that an immunopathological response elicited by this pathogenic EHV-1 strain and likely mediated by T lymphocytes results in fatality. Although the results in Fig. 1 clearly show a similar presence of CD4 and CD8 T cells within the lungs and BAL fluid of mice infected with either KyA or RacL11, they reveal nothing regarding the function of these lymphocytes. We have previously characterized the primary and memory CTL responses elicited by KyA in the draining LN and spleen, respectively (41). To date, however, there is no information regarding CTL activity directed to the RacL11 EHV-1 strain. Furthermore, primary CTL activity isolated from the infected lung and directed against either KyA or RacL11 has not been demonstrated. When lymphocytes were isolated by enzymatic digestion from the entire lung, cultured for 3 days in vitro, and tested against EHV-1-infected targets, the primary CTL responses elicited by KyA and RacL11 were identical (Fig. 3). Further, when the cytolytic activity of lymphocytes isolated from the BAL fluid at 5 days postinfection was assessed, the primary EHV-1-specific CTL responses were also very similar. The percent specific lysis at an effector-to-target ratio of 30:1 was 32 and 36% for the BAL fluid from KyA- and RacL11-infected mice, respectively (data not shown). In a previous report (41), it was observed that KyA elicited a vigorous primary CTL response in the lung draining mediastinal LN but not in the draining cervical LN, even though the cervical LN drains the nasal turbinates, an important site for initial EHV-1 replication (7). Identical findings were observed following infection with the pathogenic RacL11 strain (data not shown). Taken together, these results indicate that, by all parameters tested, the primary RacL11-specific CTL response in the draining LN and lung is identical to that observed following infection with the attenuated EHV-1 strain KyA.

Analysis of cytokine transcripts in the infected lung. The identical kinetics of viral clearance and the striking similarity of the primary CTL responses following RacL11 infection and KyA infection suggest that the massive inflammatory response within the RacL11-infected lung may reflect a difference in the profile of cytokines elicited by pathogenic versus attenuated EHV-1. RNase protection assays were performed to identify differences between the profiles of specific proinflammatory cytokines elicited by these two biologically distinct EHV-1 strains. RNase protection assays analyzing RNA isolated from the entire lung on days 3 and 5 postinfection revealed equivalent amounts of transcripts specific for interleukin 10 (IL-10) and the proinflammatory cytokines IL-15, IL-6, and gamma interferon in KyA- and RacL11-infected lungs (data not shown). However, when RNA isolated from BAL fluid on days 3 and 5 was analyzed, much higher levels of transcripts specific for the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) (Fig. 4A) and the beta chemokines macrophage inflammatory protein 1α (MIP-1α), MIP-1β, and MIP-2 (Fig. 4B) were detected on day 3 for RacL11-infected mice. Quantitation of results from four separate experiments demonstrated a consistent upregulation of MIP-1α, MIP-1β, and MIP-2 transcripts relative to the housekeeping gene L32 in EHV-1 RacL11-infected lungs (Table 2). In all four experiments, the levels of MIP transcripts from RacL11-infected mice were above the levels of L32 transcripts. In contrast, the levels of transcripts of all three MIP chemokines from KyA-infected lungs were consistently below the levels of L32 transcripts. MIP transcript levels in KyA- and RacL11-infected lungs were above the levels of L32 transcripts. In contrast, the levels of transcripts of all three MIP chemokines from KyA-infected lungs were consistently below the levels of L32 transcripts. MIP transcript levels in KyA- and RacL11-infected mice, calculated as percentages of L32 transcript levels, were compared, and the levels of MIP transcripts in RacL11-infected mice ranged from ~2- to ~7-fold greater than those in KyA-infected mice (Table 2). Interestingly, the levels of MIP-
1α, MIP-1β, and MIP-2 transcripts isolated from the BAL fluid of RacL11-infected mice decreased by day 5 postinfection, while the levels in KyA-infected mice remained relatively unchanged (Fig. 4B). These results suggest that a rapid and vigorous production of these proinflammatory chemokines plays a major role in recruiting the massive inflammatory infiltration present in RacL11-infected lungs.

DISCUSSION

Natural infections of horses with EHV-1 are associated with distinctive immunopathology of the upper respiratory tract and pathognomonic clinical signs (5). However, infection with the EHV-1 vaccine candidate strain KyA exhibits none of these signs and protects equines against subsequent infection with pathogenic, clinical isolates of EHV-1 (28). This difference between the host responses to the two viral strains suggested that the respiratory tract disease was due to immunopathological changes induced by clinical isolates rather than to differences between the immune responses to the two strains.

In CBA (H-2k) mice, clinical signs reminiscent of those in the equine are observed in response to infection with the pathogenic strain RacL11. However, the pathogenic strain RacL11 and the attenuated strain KyA reach similar levels of infectious progeny in the infected lung (41) and induce similar CD8+ T-cell responses (this study). Importantly, the two virus

### TABLE 2. Quantitation of RNase-protected transcripts

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Transcript</th>
<th>Levela in mice infected with:</th>
<th>Fold expressionb</th>
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<tr>
<td></td>
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<td>RacL11</td>
<td>KyA</td>
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<tr>
<td>1</td>
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<td>42°</td>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>MIP-2</td>
<td>193</td>
<td>32</td>
</tr>
</tbody>
</table>

a Expressed as a percentage of the L32 transcript level. The integrated density of each RNase-protected band was determined as described in Materials and Methods.

b Calculated as the fold increase in RacL11-specific transcript levels relative to KyA-specific transcript levels.

The significance of the difference between the mean values of each chemokine species in KyA- and RacL11-infected mice was determined by the Student two-tailed t test. The P values for MIP-1β, MIP-1α, and MIP-2 were all <0.005.
strains are cleared with identical kinetics, indicating that the severe clinical signs and fatality following RacL11 infection are not due specifically to viral load, but instead likely represent an immunopathological response by the host to the pathogenic strain. Further, both strains induce the infiltration of similar numbers and ratios of CD4 and CD8 T cells and induce identical EHV-1-specific CTL responses within the lung, BAL fluid, and draining LN. Therefore, the clinical outcome of RacL11 infection is not due to virus load or to the failure of the virus to induce a humoral (48) or cell-mediated immune response (41). The intense inflammatory response and DAD associated with RacL11 infection suggest that this strain has the ability to induce damaging cytokine species within the lung.

RNase protection analysis of transcripts isolated from the entire lung, either by physical grinding or by enzymatic digestion, revealed no obvious differences in the cytokines produced in the lung following infection with either strain (data not shown). However, when mRNA was isolated from the BAL fluid, the levels of MIP-1a, MIP-1b, and TNF-a transcripts on day 3 postinfection were much greater in response to RacL11 than to KyA. Early studies reported proinflammatory properties associated with these chemokines (16, 17, 39). Subsequent studies have implicated MIP-1a and MIP-1b in a variety of inflammatory diseases, including inflammatory muscle disease (1), herpes stromal keratitis (41), alcoholic hepatitis (3), and pulmonary inflammation (18, 26). Similarly, an important role for MIP-2 in pulmonary sepsis and interstitial lung disease has been demonstrated (18, 23, 45). The chemotactic properties of MIP-1a, MIP-1b, and MIP-2 for neutrophils (16, 39, 44) and monocytes (18, 43) correlate with the presence of both monocytes and neutrophils in the BAL fluid isolated from RacL11-infected lungs at 5 days postinfection. Interestingly, the levels of MIP-1a, MIP-1b, and MIP-2 transcripts are actually much lower in RacL11-infected lungs 5 days postinfection than in KyA-infected lungs. The downregulation of chemokine production by day 5 occurs when cellular infiltration is most severe and likely reflects no further need to recruit neutrophils and monocytes.

Infection of CBA mice with the pathogenic RacL11 EHV-1 strain results in primarily a lymphocytic and neutrophilic interstitial pneumonia in contrast to the lung eosinophilia observed following infection of the mouse with respiratory syncytial virus (RSV) (25, 33, 34, 38). The involvement of eosinophils following RSV infection of the lung is thought to be closely linked to the production of eosinophilic factors including RANTES and IL-5 (25, 38). Although RANTES transcripts are produced at slightly higher levels in RacL11-infected lungs than in KyA-infected lungs (Fig. 4), no appreciable differences between the levels of IL-5 transcripts were detected (data not shown).

Previous studies have demonstrated the induction of MIP-1a, MIP-1b, and MIP-2 expression by stimulation with TNF-a (8, 19, 20). The results in Fig. 4A are in agreement with this reported observation and show a concurrent upregulation of TNF-a transcripts on day 3 postinfection in RacL11-infected mice. Taken together, the results presented here suggest that proinflammatory MIP-1a, MIP-1b, and MIP-2 are produced at high levels in response to RacL11 by day 3 postinfection and are downregulated by day 5, when the inflammatory infiltration and resulting tissue damage are severe.

A clear distinction between the levels of virus in the lungs and the immunopathological destruction observed was shown recently in a study of herpes simplex virus type 1 (HSV-1) pneumonia in mice (2). It was shown that inhibition of the inducible form of nitric oxide synthetase (NOS2) reduced the immunopathology associated with pneumonia to the extent that mice survived normally lethal doses. Interestingly, the levels of HSV-1 recovered from infected lungs in mice receiving the specific inhibitor were significantly greater than those from untreated controls. Therefore, NOS2 and nitric oxide itself appear to be important for HSV-1 clearance from the lung but also have a detrimental effect, inducing the immunopathological changes associated with lethal pneumonia. It is likely that the response to EHV-1 in the lung walks the fine line between elimination of infection and induction of damaging pathology.

If the difference between the responses to KyA and RacL11 cannot be explained by differences in viral load in the lung, then RacL11 must encode a protein(s) important in eliciting the immunopathology observed. Sequence analysis of EHV-1 KyA revealed a number of deletions within open reading frames (ORFs) in the viral genome. Deletions within the unique short segment of the genome include the viral envelope glycoproteins gL and eG, an ORF encoding a 10-kDa protein (21), and a large deletion in the EUS4 ORF (15). Deletions within the unique long (UL) region of the genome include an internal in-frame deletion within the EICP0 gene (9), a 1,283-bp deletion near the UL terminus (46, 47), and a 1,207-bp deletion located 5’ to the glycoprotein C ORF (29). Presently, it is not clear which of these gene products is associated with the immunopathology observed in mice or equines. However, deletion of the ORFs encoding gL and eG rendered a pathogenic strain of EHV-1 avirulent in horses, implicating these two viral glycoproteins as potential mediators of immunopathology (27). The fact that EHV-1 RacL11 is cleared from the lungs as efficiently as KyA suggests that one or more of these viral components is associated with the induction of these proinflammatory mediators associated with EHV-1 pathogenicity. Studies to address this question are in progress.

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