Equine herpesvirus 1 (EHV-1) induces a variety of disease manifestations, including respiratory disease, abortions, and myeloencephalopathy. Several vaccines are commercially available but could not previously be distinguished by serologic testing from infection with EHV-1 (or the closely related EHV-4). Currently available vaccines are not reliably protective against the severe manifestations of the disease, including fatal myeloencephalopathy. We determined immunological parameters that can differentiate vaccinated from previously infected animals by comparing humoral and cellular EHV-1-specific responses in clinically healthy horses 10 months after vaccination. Forty-seven horses with known histories of vaccination and infection were studied, including a group of horses that survived a severe neurological outbreak 5 years prior to vaccination. Results of serum virus neutralization (SN), serum IgG isotyping, and cytokine profiling of lymphocyte subsets were compared. IgG4/7 levels strongly correlated with virus neutralization ($P < 0.0001$). IgG1/3 and SN values distinguished vaccinated/outbreak-exposed (vacc/outbreak) horses from vaccinated horses ($P < 0.05$). EHV-1-specific gamma interferon (IFN-γ)-producing CD4+ (but not CD8+) T-cell numbers were also increased in vacc/outbreak horses, which distinguished them from vaccinated horses ($P < 0.01$). IFN-α secretion was similar between all groups and independent of previous exposure or vaccination. Our data suggest that IgG isotype responses to EHV-1 are more diverse under field conditions than is revealed by experimental studies and that the current modified-live virus (MLV) vaccine induces a more restricted IgG isotype response than does natural exposure to EHV-1. Since these parameters can be assessed in a high-throughput manner, they may prove useful in screening future vaccine candidates and assessing levels of protection.

The highly infectious equine herpesvirus 1 (EHV-1) is a pathogen of increasing concern to the global equine industry. EHV-1 belongs to the same genus (Varicellovirus) as varicella-zoster virus (VZV), infectious bovine rhinotracheitis virus, and pseudorabies virus (10). The clinical signs of infection with this virus in its natural host can vary from a mild upper respiratory infection to complete quadriplegia. In pregnant mares, late-term abortion or neonatal foal death can also occur (3). Although its clinical manifestations are varied, EHV-1 is an important cause of neurological disease in horses (11). In contrast to other neurologic alphaherpesviruses, which have a tropism for neurons, EHV-1 neuropathology is largely in the vascular endothelia of arteries supplying the central nervous system, although the trigeminal ganglion is involved (9). Subsequent inflammatory responses cause a myeloencephalopathy, often requiring euthanasia. As with other herpesviruses, animals infected with EHV-1 remain latently infected for life; stress or immunosuppression can trigger viral reactivation (8). Sites of latency include the trigeminal ganglia, lymphoreticular tissues, and leukocytes (reviewed by Allen et al. [3]).

In the absence of definitive immune correlates of protection, it is uncertain how long horses are protected after natural infection or vaccination. It has been suggested that the period of resistance to reinfection increases with repeated infections (38). Based on data from one of the largest neurologic outbreaks, older horses (>10 years) are more susceptible to the neurologic disease manifestations than younger horses or foals (18).

There are two high-antigen-content commercial vaccines (both inactivated) available for protection from the respiratory and abortion outcomes of infection, but neither vaccine is labeled as being effective for preventing the neurologic form. The available inactivated vaccines are mostly effective at generating serum virus neutralization (SN) responses, possibly due to the feasibility of SN testing in the vaccine screening process. In addition, one modified-live virus (MLV) vaccine is available in North America, and it confers a high level of protection against fever and other clinical signs of disease but is not labeled for use in pregnant mares (12, 13). No vaccine is completely effective at eliminating nasopharyngeal virus shedding or cell-associated viremia, which are the detectable parameters of most concern.

Rapid quantitative PCR (qPCR) testing for EHV-1 in nasal swabs and blood samples is now standard practice for the diagnosis of active infections (33). Except by nested PCR on cultured peripheral blood mononuclear cells (PBMC) (6, 44), or preferably lymph node tissues collected under general anesthesia (2), it is difficult to diagnose latent infections in live animals by molecular testing. It can be expected that the majority of horses in the United States and the United Kingdom have been exposed (25). Molecular-based tests, however, cannot determine the level of protective immunity in a clinically healthy horse, and nothing can be said about immunity to reactivation.
or reinflection based on these tests. Given recent improvements in the techniques available for genetic modification of herpesvirus (29, 37) and their potential contributions to vaccine development, enhanced diagnostic techniques for the evaluation of immunity to EHV-1 during disease outbreaks or vaccine studies are critical.

The traditional method for serologic diagnosis of EHV-1 is the SN assay, which does not clearly distinguish titers of vaccinated versus infected infected animals. However, SN titers during experimental infection or vaccination studies have been correlated with protection from some clinical signs of disease (12, 13). A further complication is that EHV-1 and EHV-4 can cross-react in SN testing (15). To determine if animals are at risk, more-complete immunological profiling is necessary, combining serological and cellular markers.

Although not attributed to one specific type of immune response, protection from EHV-1 respiratory disease has been associated with gamma interferon (IFN-γ) expression as a marker of cell-mediated immunity (reviewed by Kydd et al. [22]). Paillot et al. showed in vitro that the percentage of cells synthesizing IFN-γ is negligible in foals but increases with age (30) or vaccination with live-virus vector construct (32). Experimental infections induce IFN-γ during the viremic phase, which is also the critical period for onset of neurologic signs (4, 30). These IFN-γ-positive cells mostly display CD8+ and CD4+ phenotypes; CD8+ cells particularly have been associated with cytotoxicity against EHV-1 (1, 28). Functional assays for EHV-1 cell-mediated immunity are well established (1, 23, 26) and are based on radioisotope assays of lymphoproliferation and cytotoxic T-lymphocyte precursor levels, but these methods are not feasible for routine diagnosis of infection status or vaccine efficacy. One report showed a good correlation of EHV-1-specific cytotoxic T-cell precursor activity and IFN-γ-producing CD8+ T cells (30).

EHV-specific IgG isotyping, first performed on nasal washes in a study reported by Breathnach et al. (5), has been adapted to assess the isotypes and relative abundances of EHV-1-specific antibodies in horse sera in a nonhazardous and efficient manner (13). We previously showed that an inactivated vaccine in horses vaccinated under field conditions, including a group of horses that experienced a severe equine herpesvirus myeloencephalopathy (EHM) outbreak prior to vaccination.

### MATERIALS AND METHODS

**Horses and samples.** All animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee and were in accordance with federal guidelines. A total of 47 clinically healthy horses from Cornell equine research herds were included in the study (Table 1). Forty-one of the horses were in a herd that received a modified-live virus vaccine (Rhinomune; Pfizer Animal Health) at the recommended yearly interval; 6 horses were from a herd with no history of vaccination or infection (unvaccinated group). The unvaccinated horses were 6 to 9 years of age (median, 7 years). One was a thoroughbred and 5 were draft horse/Arabian crosses. Of the horses in the vaccinated herd, 19 experienced a severe neurological outbreak of EHV-1 in 2005 prior to their first vaccination (vacc/outbreak group). The outbreak was confirmed by clinical histories (including two horses requiring euthanasia) and diagnostic testing. Nineteen horses were not present during the outbreak (vaccinated group). Three horses had been added to the herd recently and had no history of infection (these three horses were excluded from the group analyses). The horses of the vaccinated group were 4 to 16 years of age (median, 6 years); seven were thoroughbreds, 6 were ponies, and 7 were of unknown breed. The vacc/outbreak group was composed of 12 thoroughbreds, 6 ponies, and 1 standardbred; horses were 7 to 23 years of age (median, 16 years). The MLV vaccine was last administered 9 to 10 months prior to blood sample collections; two pregnant horses in the vacc/outbreak group additionally received inactivated vaccine (Pneumabort K; Pfizer Animal Health) 2 months prior to the sampling. Blood samples were collected in January and February 2011. PBMC were isolated from all horses by density gradient centrifugation of heparinized blood (Ficoll-Paque Plus; GE Healthcare, Piscataway, NJ). Sera from coagulated blood samples were collected for EHV-1-specific SN and IgG isotype analyses.

**SN assay.** Sera were assayed for EHV-1 neutralization by a standard microneutralization assay (7, 36) at the Animal Health Diagnostic Center at Cornell University (control strain provided by G. Allen, University of Kentucky). Virus and serum were incubated for 1.5 h prior to inoculation on RK13 cells, and the 50% neutralizing dilution was determined.

**EHV-1 IgG isotype-specific ELISA.** IgG isotyping was performed as previously described (13, 42). Enzyme-linked immunosorbent assay (ELISA) plates were coated with purified EHV-1 virions (5 μg/ml) and incubated overnight at 4°C. Five washing steps were performed between all incubations. The equine serum samples, including four serum controls ranging from negative to high positive based on preliminary testing, were diluted 1:200 and incubated for 1 h at 37°C. Plates were incubated for 1 h with the primary monoclonal antibodies (Table 2) at a 1:20 dilution, followed by detection with a secondary peroxidase-conjugated anti-mouse IgG (H+L) antibody (Jackson ImmunoResearch, West Grove, PA) at 1:20,000. Kinetic plate reads were obtained at 45, 90, and 135 s after the addition of substrate in an ELISA reader (ELx808; Biotek Instruments Inc., Winooski, VT) for absorbance at 630 nm. The three measurements

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of horses</th>
<th>Age range (yrs)</th>
<th>Breeds</th>
<th>Time since last known EHV-1 exposure</th>
<th>Time (median) since last EHV-1 vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unvaccinated</td>
<td>6</td>
<td>6–9 (median, 7)</td>
<td>1 thoroughbred, 5 draft horse/Arabian crosses</td>
<td>NA*</td>
<td>NA</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>20</td>
<td>4–16 (median, 6)</td>
<td>7 thoroughbreds, 3 ponies, 7 unknown</td>
<td>NA</td>
<td>9–10 mos (10 mos)</td>
</tr>
<tr>
<td>Vaccinated/outbreak group</td>
<td>18</td>
<td>7–23 (median, 16)</td>
<td>12 thoroughbreds, 6 ponies, 1 standardbred</td>
<td>5 yrs</td>
<td>2–10 mos (10 mos)</td>
</tr>
</tbody>
</table>

*NA, not applicable.*
TABLE 2 Primary monoclonal antibodies used for serum IgG isotyping

<table>
<thead>
<tr>
<th>Antibody</th>
<th>IgG subtype recognized</th>
<th>Original nomenclature</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVS 39</td>
<td>IgG4, IgG7</td>
<td>IgGb</td>
<td>24, 34</td>
</tr>
<tr>
<td>CVS 40</td>
<td>IgG3, IgG5</td>
<td>IgG(T)</td>
<td></td>
</tr>
<tr>
<td>CVS 45</td>
<td>IgG1</td>
<td>IgGa</td>
<td></td>
</tr>
<tr>
<td>267</td>
<td>IgG6</td>
<td>IgGc</td>
<td>12</td>
</tr>
<tr>
<td>416</td>
<td>IgG5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>159</td>
<td>IgG1/3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* IgG subclasses are denoted by the current genetic classifications, based on heavy-chain constant-region genes (39). The original nomenclature, based on electrophoretic mobility, is also indicated.

created a linear curve and a slope value for each sample and IgG isotype. The slope value was multiplied by 1,000 for data presentation.

**Cytokine secretion assay.** EHV-1 infection of PBMC and cytokine detection in supernatants were performed as previously described (43). In brief, a total of 6 × 10⁶ PBMC per well were cultured in 200 μl of cell culture medium (Dulbecco’s modified Eagle’s medium [DMEM] containing 10% [vol/vol] fetal calf serum, 1% [vol/vol] nonessential amino acids, 2 mM l-glutamine, 50 μM 2-mercaptoethanol, 50 μg/ml gentamicin; all from Gibco, Invitrogen, Grand Island, NY) in 96-well plates. PBMC were cultured for 48 h in a 5% CO₂ incubator at 37°C. Cells either were kept in medium or were infected with the EHV-1 strain RacL11 at a multiplicity of infection (MOI) of 1. RacL11 is the virulent laboratory strain of EHV-1 from which the Rhinomune MLV strain RacH was derived (19). Interleukin-4 (IL-4), IL-10, IL-17, IFN-γ, and IFN-α were simultaneously measured in cell culture supernatants from EHV-1-infected PBMC and noninfected cells by using a fluorescent bead-based multiplex assay described previously in detail (41). IL-4, IL-10, and IFN-α results are reported in pg/ml, and IL-17 and IFN-γ levels are reported in U/ml.

**T-cell phenotyping and cytokine profiling by flow cytometry.** A total of 3 × 10⁶ equine PBMC were cultured for 48 h in DMEM as described above. Cells were stimulated with phorbol myristate acetate-ionomycin or infected at an MOI of 1. Nonstimulated controls were cultured in medium. Brefeldin A was added to all samples at 24 h. Cells were fixed, permeabilized, and stained for CD4 or CD8 and for IFN-γ, IL-4, or IL-10 expression by using directly labeled antibodies and isotype-matched staining controls as previously described (40). The cells were analyzed in a FACSCanto II flow cytometer (BD Biosciences, San Diego, CA), with fluorescence gates set according to the isotype control staining.

**Statistics.** Data were analyzed using the GraphPad Prism program, version 5.01. Kruskal-Wallis tests were performed for assessments of differences between groups. Spearman correlations were performed for analysis of associations between SN or IFN-γ and all other parameters. Data were considered significant if P values were <0.05, with P levels of <0.01 and <0.001 further denoted. In all figures, the circle symbols represent data from individual horses, with horizontal lines denoting group medians. The box-and-whisker plots (as used for Fig. 2A and 4A and B) denote quartiles (in the box) and minimum/maximum values (the whiskers).

**RESULTS AND DISCUSSION**

**SN antibody response to EHV-1.** The median SN titers for unvaccinated, vaccinated, and vac/outbreak animals were 8, 64, and 192, respectively (Fig. 1). Vaccinated (P < 0.05) and vac/outbreak animals (P < 0.001) had significantly higher SN titers than unvaccinated horses. Vac/outbreak horses also had higher titers than vaccinated horses (P < 0.05).

Directed at EHV-1 surface glycoproteins, SN antibodies play a role in reducing the spread of viruses released from cells. Since EHV-1 can spread directly between lymphocytes, neutralizing antibodios are likely most beneficial during the early stages of viral replication in respiratory epithelia, where they have been shown to reduce nasal shedding, but may have little effect on cell-associated viremia or abortion (14, 17, 27). Because EHV-1 outbreaks occur despite wide use of vaccination, the value of EHV-1-specific SN titers as indicators of overall protective immunity remains questionable.

**EHV-1-specific IgG isotypes after natural infection or vaccination.** The most prevalent serum IgG isotype in all horses investigated here was IgG4/7 (IgGb) (Fig. 2A). The IgG4/7 level was the only IgG isotype measurement that could significantly distinguish vaccinated and unvaccinated horses (P < 0.05) (Fig. 2C). IgG6 and IgG1 were almost undetectable in all samples (Fig. 2A). According to Soboll-Hussey et al. (35), horses experimentally infected with a neuropathogenic EHV-1 strain produced almost no IgG3/5, short-lived IgG1 and IgG6, and strong IgG4/7 responses that mimicked the increase in SN titers. Our testing for IgG1/3, IgG4/7, and IgG5 in healthy horses differentiated vac/outbreak from unvaccinated horses with a high level of significance (P ≤ 0.01).

In _in vivo_ studies have revealed that EHV-1-specific IgG4/7 antibodies can differentiate protected from unprotected animals following EHV-1 challenge. IgG3/5 has either been correlated with increased clinical disease and/or viral shedding or has not been detected in experimental infection studies (13, 35). High IgG3/5 values have been detected in vaccinated horses (MLV and inactivated), with the highest response from a high antigen load inactivated vaccine (Pneumabort-K; Pfizer Animal Health) (12).

Here, we observed similar trends in IgG isotype profiles as previously reported; i.e., vaccination with an MLV vaccine induced IgG4/7 antibodies. IgG4/7 was the dominant isotype of the antibody response in all horses. In vac/outbreak horses, the EHV-1-specific IgG isotype values were overall higher than in the vaccinated group. This suggests that IgG isotype responses to EHV-1 are more diverse under field conditions than revealed by experimental infection studies and that current vaccines induce a more restricted IgG isotype response than does natural exposure to EHV-1. It also suggests that a more diverse IgG isotype response to EHV-1 is beneficial, as long as IgG4/7 is the dominating antibody isotype, because the vac/
outbreak horses in this study survived a severe EHV-1 outbreak in which several horses died.

**Type I and II interferon induction by EHV-1.** EHV-1 infection of PBMC induced significant amounts of IFN-α and IFN-γ compared to noninfected cells (Fig. 3A and B). IL-4, IL-10, and IL-17A were not expressed above background levels in any of the horses tested (data not shown).

IFN-α is a cytokine of the innate immune response. It is induced by interaction of viral components, such as double-stranded DNA, with host cell pattern recognition receptors (20). EHV-1-induced IFN-α secretion did not differ between unvaccinated, vaccinated, and vacc/outbreak horses (Fig. 3C), although the median IFN-α secretion level from EHV-1-infected PBMC of the unvaccinated horses was lower than in the vaccinated or vacc/outbreak groups. In equine PBMC, IFN-α secretion was previously described after infection with EHV-1 in vitro (43). Viral interaction with pattern recognition receptors does not require preexisting adaptive cellular immunity, and it was thus not sur-
The data suggest that vaccination with the commercial MLV vaccine induced cellular immune responses inconsistently. This may be a reflection of the animals' variable but unknown infection histories. Natural infection prior to vaccination resulted in robust numbers of IFN-γ+ T cells in almost all horses. The data also suggest that Th1 cells (CD4+/IFN-γ+) may play an important role during natural infection with EHV-1. Considering that the horses in the vacc/outbreak group survived a severe EHM outbreak, these EHV-1-specific Th1 cells could be interpreted as markers of immunity. Previous studies investigating adaptive immune responses to EHV-1 suggested that increased preinfection levels of EHV-1-specific cytotoxic T lymphocytes were protective from progression to respiratory or abortigenic disease (1, 23). We did not observe a significant increase in EHV-1-specific CD8+ responses in the horses investigated here under field conditions. Paillot et al. (31) also observed that the majority of IFN-γ-producing lymphocytes stimulated by EHV-1 in older ponies (not recently infected) were CD8 negative. This discrepancy could result from the assay used to assess the cellular immune response, the time between infection and sampling, the virus strain, or other differences in study designs. Our findings of increased Th1 cell numbers after natural infection with EHV-1 are in agreement with CD4-mediated immunity to VZV, a neurotropic alphaherpesvirus of humans. The classic manifestation of zoster disease is due to loss of T-cell-mediated immunity despite the continued presence of antibodies to VZV (16). Although EHV-1 is not associated with neuronal infection, it shares essential properties with the other alphaherpesviruses, including the ability to downmodulate immune responses, and could potentially be prevented by a CD4-mediated response.

**Correlations of all immunological parameters.** All parameters tested here were significantly correlated with SN values (P < 0.01), with the exception of IgG6. The Spearman rank correlation coefficient for SN and IFN-γ was 0.51 (P = 0.0003). In accordance with the findings reported by Soboll-Hussey et al. (35), IgG4/7 was strongly correlated with SN (0.65/P < 0.0001) but not with IFN-γ production. Similarly, Goehring et al. (12) observed correlations...
of IgG1 and IgG4/7 with SN patterns following a high-dose experimental infection with a neuropathogenic strain of EHV-1. Here, IFN-γ was significantly correlated with all tests, with the exceptions of IgG6 and IgG4/7.

Conclusions. Due to the cell-associated viremia that is central to the in vivo infection process of EHV-1, cell-mediated immunity is considered critical in EHV-1 protection (21, 22, 31). As with most viral infections, a Th1-polarized immune response associated with high levels of EHV-1-specific IFN-γ production likely contributes to protection against disease. In previous studies, CD8+ IFN-γ+ cells were shown to control cell-associated viremia and disease, although not specifically the neurological outcome. Here, we observed elevated Th1 responses in almost all horses that survived a severe EHM outbreak.

At this point, both cellular and humoral factors should be considered in assessments of protective immunity against EHV-1. Specifically, the combination of IFN-γ detection, T-cell phenotyping, IgG isotypes, and SN testing will give a more comprehensive picture of immunity and should be included in the vaccine screening process.

ACKNOWLEDGMENTS

This work was supported by a grant from the Harry M. Zweig Memorial Fund for Equine Research to B.W. and by NIH fellowship F32 AI082922 to L.B.G.

We thank D. Antczak and the reviewers for critically reading the manuscript.

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