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Report of the equine herpesvirus-1 Havermeier Workshop, San Gimignano, Tuscany, June 2004

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Abstract

Amongst the infectious diseases that threaten equine health, herpesviral infections remain a world wide cause of serious morbidity and mortality. Equine herpesvirus-1 infection is the most important pathogen, causing an array of disorders including epidemic respiratory disease abortion, neonatal foal death, myeloencephalopathy and chorioretinopathy. Despite intense

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scientific investigation, extensive use of vaccination, and established codes of practice for control of disease outbreaks, infection and disease remain common. While equine herpesvirus-1 infection remains a daunting challenge for immunoprophylaxis, many critical advances in equine immunology have resulted in studies of this virus, particularly related to MHC-restricted cytotoxicity in the horse.

A workshop was convened in San Gimignano, Tuscany, Italy in June 2004, to bring together clinical and basic researchers in the field of equine herpesvirus-1 study to discuss the latest advances and future prospects for improving our understanding of these diseases, and equine immunity to herpesviral infection. This report highlights the new information that was the focus of this workshop, and is intended to summarize this material and identify the critical questions in the field.

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1. Introduction

The equid herpesviruses (EHVs) are major equine pathogens and have been subject to a concerted international research effort for the last five decades. Of the nine EHVs that have been described, two of these, EHV-1 and EHV-4, have been the source of most concern to the horse industry. Both are transmitted via the respiratory route and infect the epithelium of the upper respiratory tract, although there is accumulating evidence that EHV-1 may not be a cause of clinically-important respiratory disease. Both viruses establish long-lived, possibly life-long, latent infections in recovered horses which act, via periodic reactivation, as reservoirs of infection for new, susceptible horses. EHV-1 causes a cell-associated viremia and is responsible for abortions and neurological disease. EHV-4 generally does not cause viremia and hence EHV-4 abortions and neurological disease are rare. It has been recognised for many years that EHV-1 isolates vary in pathogenicity with 'high' and 'low' virulence isolates circulating in the horse population. Researchers now possess many of the immunological and genetic tools required to dissect the pathogenesis and immune control of EHV infections. Over the last decade considerable advances have been made in this regard and it is now clear that control of infection requires a complex, multi-component immune response. This paper presents the results of a workshop on EHV-1 immunology that was held in Tuscany, Italy in June 2004.

2. Epidemiology and clinical disease

A more complete understanding of the epidemiology of EHV-1 and a detailed appreciation of pathogenesis

has provided insight into the complex and multi-component immune response required to control infection (Allen et al., 1998). It is now clear that immunological control of pathogenesis requires a coordinated response from mucosal and systemic immune systems involving both humoral and cell mediated immunity (Allen et al., 1998; O'Neill et al., 1999; Soboll et al., 2003; Kydd et al., 2003). The epidemiology and pathogenesis of abortion and neurological disease illustrate the difficulties faced by the immune system in controlling EHV-1 infections and provide insight into the goals for future effective vaccines. The pathogenesis of neurological disease in the USA and Europe was reviewed in detail in several presentations. The clinical presentation, diagnosis and management of the EHV-1 outbreak at the University of Findlay provided valuable new insight into virus transmission from neurological cases: infection with EHV-1 in adult horses did not cause clinically-apparent respiratory disease; only a proportion of infected horses developed neurological signs; and contagion occurred from horses with neurological disease (Reed, OH, USA). There were few obvious clinical signs to suggest that EHV-1 had entered the yard: initial clinical signs were vague, consisting of pyrexia and depression affecting 87% of the population of 135 horses. Neurological clinical cases, many of which were severely affected recumbent horses with complications including vasculitis, urinary incontinence and penile paralysis, developed over the next 2 weeks. Overall 42 horses (34%) developed neurological disease. Management was based on the assumption that these cases were not contagious and recumbent horses were hospitalised at the Ohio State University (Kohn, OH, USA) and not isolated. Within 12 days, in-contact horses in the hospital developed pyrexia; three of these developed

neurological disease and were virus isolation and PCR positive on nasopharyngeal swabs. Two further horses with pyrexia seroconverted but did not develop clinical disease. There was speculation that EHV-1/-4 vaccination may contribute to the frequency and severity of clinical disease since a greater proportion of horses vaccinated three to four times per year (40%) developed neurological disease compared to those vaccinated one to two times per year (22%). An EHV-1 outbreak in a Belgian riding school had also caused severe clinical disease (van der Meullen, Ghent, Belgium). Horses developed either pyrexia followed by progressive ataxia or acute onset paralysis, in some cases (24%) with cerebral signs. This outbreak resulted in high mortality: death or euthanasia occurred in 15%. As in the Ohio outbreak, pyrexia was the major clinical sign before the onset of neurological disease, only a proportion of horses with pyrexia developed neurological signs and there was contagion to in-contact premises suggesting that affected horses shed infectious virus. Preventing or limiting EHV-1 cell-associated viremia is regarded as a central requirement of protective immunity (Allen et al., 1998) and the generation of virus-specific cytotoxic T lymphocytes (CTL) is a critical immune effector in the control of EHV-1 disease (Allen et al., 1995; O'Neill et al., 1999; Kydd et al., 2003). The identity of virus-infected cells in the circulation cells appears to be principally mononuclear cells but immune targeting of these cells is compromised because infected peripheral blood mononuclear cells (PBMC) generally do not express virus antigens on their surface (van der Meullen, Ghent, Belgium). Nine outbreaks of neurological disease that had occurred in Holland between 1999 and 2003 provided some insight into the role of viremia in the pathogenesis of neurological disease (Goehring, Utrecht, The Netherlands). As in the USA and Belgian outbreaks, pyrexia was the only major clinical sign that preceded neurological disease. Real time PCR has been employed to quantitate viremia by measuring virus DNA load in PBMC, potentially adding further information about the role of viremia in development of disease (Goehring, Utrecht, The Netherlands). Data from an experimental EHV-1 challenge suggested that virus load during viremia may not be the sole determinant of clinical outcome since viremia was not detected in 3/4 horses that developed neurological disease. Real time PCR measuring DNA for the virus gene encoding glycoprotein B (gB) as well as gB mRNA

transcripts and the 'latency associated transcript' (LAT) was employed to investigate a natural outbreak of EHV-4 in 11 young horses (Wilson, CA, USA). Virus DNA was still present in nasal secretions at day 28 (9/11 horses) and although blood was positive (11/11) on day 1, blood from all 11 horses was negative after day 14. Similarly, LAT mRNA was not detected in blood samples after day 14 suggesting that latency was not established in circulating PBMC.

The pathogenesis of EHV-1 abortion involves virus translocation from the circulation into the placental unit and induction of uterovascular lesions. The key step is likely to be infection of placental endothelial cells and it may be that these cells present brief targets for the immune system because they transiently express viral antigens (Smith, Newmarket, UK) detectable by immunoperoxidase staining of histology sections. In a study of 49 field abortions (Smith et al., 2003), endothelial cell infection occurred mainly in microcotyledonary arterioles although there was often no associated thrombosis. Vasculitis was most pronounced in months 5–9 of gestation suggesting a possible role of gestational age or other host factors in pathogenesis (Smith et al., 1996). Infection of the foetus is not a prerequisite for abortion, however, since spread of virus to the foetus via endometrial villi occurs after microcotyledonary infection (Smith et al., 1992). Between 2001 and 2003, the AHT investigated 241 UK abortions. Of these 9 were 'typical' EHV-1 abortions, i.e. the placenta and foetus were virus positive, whilst 6 were 'atypical' where there was no detectable foetal infection (assessed by PCR and immunoperoxidase methods).

The genome sequence (Telford et al., 1992) of EHV-1 has facilitated molecular epidemiological studies. The genome sequences of two contrasting EHV-1 strains (Tearle et al., 2003) have been compared (Davis-Poynter, Newmarket, UK) allowing sequence variations that may account for phenotypic differences to be identified. Comparison of Ab4 (an endotheliotropic, abortigenic and paralytic virus which causes 'high level' viremia) and V592 (a less virulent virus that does not cause paralysis and is associated with 'low level' viremia and reduced endotheliotropism and abortigenic potential) revealed 0.1% difference in sequence (150 bases in 150,000), mostly single base changes, causing coding changes in 31 open reading frames (ORFs). Of these, ORF 68

(Us2) showed the highest variation rate (2%) and was used as a phylogenetic marker to separate an international collection of viruses from Europe and the USA into six groups. This has allowed molecular epidemiology from a number of outbreaks to be carried out. For example, the five viruses isolated from the Findlay and the Ohio State University outbreak all belonged to the same ORF 68 group (group 2); other outbreaks in the USA (Pennsylvania and Kentucky) and UK (Kent) were caused by different viruses. This approach provides a means of genetic typing which can be refined by including other ORFs that segregate with ORF 68. There does not appear to be any relationship between the phylogenetic groups generated by ORF 68 analysis and the paralytic potential of strains. However, the sequence variation of the DNA polymerase gene encoded by ORF 30 does provide a means of identifying paralytic viruses (Davis-Poynter, Newmarket, UK). There are two single base changes between Ab4 and V592: G becomes A at positions 2254 (altering the amino acid at position 752 from D to A) and 2968. The majority of non-paralytic strains (69/71) examined possessed A at position 2254 whilst the majority of paralytic viruses (35/41) possessed G at position 2254. Although virulence may intuitively be expected to be determined multigenically, these data suggest that dimorphism within the DNA polymerase gene correlates with paralytic or non-paralytic potential.

3. Virology

Since the first complete EHV-1 genome was sequenced and published (Telford et al., 1992) sequencing technology has advanced considerably and obtaining partial or complete virus genome sequences is now comparatively rapid and straightforward. The genome of EHV-4 has been sequenced (Telford et al., 1998) and sequencing entire genomes of EHV-1 of interest is now a practical prospect. The availability of genome sequence, together with the molecular techniques and reagents to manipulate individual or groups of genes, has revolutionised understanding of EHV-1 gene function and regulation. This has major implications for EHV-1 immunology since elucidation of virus gene expression is likely to provide essential insight into virus

antigen targets for the immune system. An overview of EHV-1 gene regulation was provided by Dennis O'Callaghan (LA, USA). The 155 kb genome encodes 76 genes of which 63 are located in the unique long (U_L) region, 9 in the unique short (Us) region and 6 in the internal repeat (IRs) regions. Gene transcription and regulation is tightly and sequentially regulated into three phases: immediate early (IE), early (E) and late (L). The virus encodes a single IE gene, 55 E genes and 20 L genes. Six of these (IE, $E \times 4$ and $L \times 1$) have regulatory functions and are responsible for the tightly controlled cascade of virus gene regulation. The single IE protein is key from an immunological perspective since it is the first virus expressed by infected cells and has been identified as a target for CTL activity (Soboll et al., 2003). The 1487 amino acid IE protein is essential for virus replication and is a vital regulatory protein (Buczynski et al., 1999) repressing its own promoter and trans-activating expression of early and late gene promoters (Smith et al., 1992, 1995). Mapping the functional domains of the IE protein has been carried out using a series of partial deletion mutants revealing that 21% of the ORF is essential, amino acids 3–89 are the DNA binding domain responsible for binding its own promoter, amino acids 963–970 are responsible for translocation of the IE protein to the infected cell nucleus and amino acids 407–757 bind transcription factor IIB (TFIIB) (Albrecht et al., 2003). Four E proteins (EICP22, 27, 0 and TR2) have regulatory activities. EICP22 (Holden et al., 1995) and 27 cooperate with the IE protein (Albrecht et al., 2005) to trans-activate E and L genes whilst the early protein EICP0 is also a powerful trans-activator (Kim et al., 2004) but is antagonistic to the IE protein and competes for TFIIB. The late protein regulatory protein ETIF is the product of U_L48 (Kim and O'Callaghan, 2001). It is the EHV-1 equivalent of the herpes simplex virus alpha trans-inducing factor (α TIF) and possesses a trans-activating domain that binds upstream of the IE gene (Osterreider, NY, USA). ETIF is required for cell-to-cell spread: ETIF mutants produce small plaques in cell culture and although capsids are produced, envelopment in the cytoplasm does not occur. Mutagenesis studies carried out using bacterial artificial chromosome (BAC) technology has allowed identification of IE, IR2, EICP27, EICP22 and ETIF as essential genes.

Further presentations focussed on the characterisation of individual EHV-1 genes, especially the surface glycoproteins, and their products, in particular the use of BAC technology and mutagenesis to elucidate gene function. The U_L22 gene product glycoprotein gH has been compared in EHV-1 and EHV-4 (Neubauer, Munich, Germany). The protein is a membrane anchored E/L protein and has different sizes in EHV-1 and EHV-4 (849 and 856 amino acids, respectively) with 85.7% identity between the two viruses. Studies in BAC pRACL11ΔgH showed that the altered plaque phenotype of the mutant was restored by EHV-1 gH but not EHV-4 gH suggesting that although the two proteins are similar they are not complementary. The late gene ORF 71, encoding glycoprotein gD, may be involved in virulence, host cell tropism and virus egress from infected cells (Osterreider, NY, USA). Comparison of the virulent strain RacL11 and the hamster passage attenuated strain KyA revealed a 1242 bp deletion in ORF 71. gD mutants have slightly decreased plaque size and are less virulent to mice. gD may also be involved in determining cell tropism for EHV-1 (Whalley, Sydney, Australia); EHV-1 gD is essential for EHV-1 entry into RK cells and expression of EHV-1 gD on the surface of RK cells renders them susceptible to EHV-4 infection raising the possibility that gD is one of the virus components responsible for the differences in cell tropism between EHV-1, which has a wide cellular tropism, and EHV-4, which has a narrow cellular tropism. BAC technology provides new opportunities for genetic characterisation of EHV-1 isolates (Osterreider, NY, USA). Infectious clones have been generated for 'old' and 'new' abortigenic and paralytic strains Ab4, V592, NY03 and VA03 which will allow both targeted and random mutagenesis studies to be carried out in a highly controllable system. The genome of strain HVS25 has been examined using random Mu transposon mutagenesis and the EHV-1 BAC (Whalley, Sydney, Australia). The large library of mutants generated contained mutants in all 11 glycoprotein genes. These were analysed for cell growth and tropism, allowing the functional significance of specific domains within the genes to be identified. It is likely that the BAC system will be a valuable tool for dissection of the genetic basis of virulence via library screening for changes in phenotypes of interest, via gene transfer studies

between isolates with different pathogenic phenotypes and by identification of antibody epitope regions. BAC technology may provide a platform for future vaccine development.

4. Immunology

The discussion of immunity to EHV-1 began with a plenary presentation from Julia Kydd (Newmarket, UK). For the first four decades, immunological research efforts concentrated on the systemic humoral responses to EHV-1 infection. The last decade has seen major advances in EHV-1 immunology with the development of methods to measure the cellular immune response to infection and appreciation of the importance of mucosal immune responses. Much of these data have come from experimental infections and there remains a paucity of information from field infections or naturally-occurring outbreaks. The state-of-the-art has advanced rapidly so that mapping the viral epitopes that drive CTL responses and dissection of the interactions between MHC haplotype and virus antigen recognition by CTL are now possible using highly controllable *in vitro* systems.

The systemic humoral responses to infection have been thoroughly characterised. Infection induces both short lived (<3 months) complement fixing (CF) and longer lived (>12 months) virus neutralising (VN) antibodies, directed mainly against the viral glycoproteins gB and gC. The principle antibody isotype responses are IgM and IgG_a, b, and c; only small quantities of IgA are detectable in the circulation. It is now clear that VN antibody titres do not correlate with protection from infection and although higher titres are associated with reduced nasal virus excretion, they do not influence viremia (Hannant et al., 1993) presumably because viremic cells express few, if any, virus antigens and do not present targets for the immune system. Mucosal IgA is produced after primary and subsequent infections (Breathnach et al., 2001) and, in contrast, titres do correlate with protection from infection. The IgA response is short lived after a single infection but persists longer with subsequent infections. It seems likely that mucosal IgA is an important first line of defence against EHV-1 infection since it is known to be neutralising *in vitro*, although its function *in vivo* has not been characterised. Understanding of the cellular

immune response has advanced at great speed in the last 10 years. In the horse, EHV-1 infection causes changes in leukocyte populations in both circulatory and pulmonary compartments with lymphopenia (CD8+ depletion) and neutropenia between days 9–13 p.i. (Kydd et al., 1996). EHV-1 viremia is associated mainly with infection of CD8+ cells although a small proportion of monocytes and possibly CD4+ cells also become infected and IFN- γ profiling suggests that both CD8+ and CD4+ cells are responsive to EHV-1 (Breathnach, KY, USA). In vitro, EHV-1 replicates in stimulated lymphocytes (Thomson and Mumford, 1977) and also infects dendritic cells (Siedeck et al., 1999) and MHC-1 expression is down-regulated on the surface of infected cells (Rappocciolo et al., 2003). The ability to study CTL responses was a major breakthrough but quickly led to the realisation that although bulk lymphoproliferative responses increased following infection there was no clear correlation with protection (Allen et al., 1995). The assay was refined by measuring the frequency of CTL precursors (CTLp) by limiting dilution analysis (LDA). This, for the first time, provided a correlate of protective immunity: high numbers of CTLp correlated with protection whereas horses with low CTLp numbers were susceptible to infection (O'Neill et al., 1999). High frequencies of CTLp also correlate with protection from abortion in the face of EHV-1 challenge (Kydd et al., 2003). Mucosal cytotoxic activity against EHV-1 has been measured ex vivo in ponies experimentally infected with EHV-1 providing the first evidence for CTL activity within the naso-pharyngeal tonsil (Breathnach, KY, USA). The techniques for in vitro characterisation of CTL responses are now sufficiently advanced for the viral antigens that act as CTL targets to be mapped (Soboll et al., 2003) with emerging technologies, e.g. MHC-1 tetramers providing the promise of fine mapping of CTL epitopes, unravelling the interactions between MHC haplotype, virus recognition and targeting by the cellular immune system.

It is now possible to state with some certainty that effective vaccines against EHV will need a safe and efficient delivery route and to induce both systemic and mucosal immune responses with a high frequency of CTLp and serum and mucosal VN antibody. This is no small demand, not least because MHC haplotype influences CTL recognition of virus antigens (Soboll et al., 2003), virus strain variation may have an effect

and the horse's immune status and pre-existing latent infection are also likely to influence the response to vaccination. Each of the current range of EHV-1 vaccines induces some, but not all, of the desired components of the immune response against EHV-1. A variety of vaccine types have now been tested in horses (Kydd, Newmarket, UK): attenuated live vaccines eliminated nasal virus shedding and viremia in one trial but did not induce mucosal immunity (Breathnach et al., 2001); virus vectors carrying EHV-1 glycoproteins gB and gC (e.g. canarypox and vaccinia; Minke, Lyon, France) induced CF and VN antibody and decreased nasal shedding but did not eliminate viremia; modified live viruses (e.g. gD and gM deletion mutants) induced CF and VN antibody and decreased nasal shedding and duration of viremia; inactivated virus vaccines induced good CF and VN titres and decreased nasal shedding and viremia but failed to induce mucosal antibody and although showed apparent protection against abortion in a pregnant mare challenge, the mares that foaled successfully had high CTLp before vaccination began possibly indicating pre-existing immunity (Kydd et al., 2003).

Further presentations on immunity covered the identification of virus antigens that stimulate CTL responses. Using dendritic cells transfected with plasmids encoding EHV-1 IE, U_L5, gB, gC, gD, gE, gH, gI or gL and CTL precursors collected from six ponies with different MHC 1 (ELA-A) haplotypes that had been experimentally infected with EHV-1, it was possible to identify virus antigens that contain CTL epitopes and their MHC 1 restriction (Lunn, CO, USA). Cytotoxic activity was MHC restricted and the EHV-1 IE protein induced CTL responses in ponies with the ELA-A3.1 haplotype. Further experiments have been carried out to identify EHV-1 proteins acting as CTL targets in ponies with the A3.1 haplotype expressing the B2 gene infected with EHV-1 (Kydd, Newmarket, UK). Mouse cells co-transfected with the equine MHC 1 B2 gene and individual EHV-1 genes 2 (unknown function), 12 (tegument), 14 (unknown function), 33 (capsid assembly), 35 (gB), 60/65 (host range factor), 63 (transcriptional activator), 64 (IE) and 69 (protein kinase) were used as targets in cytotoxicity assays. Cytotoxicity occurred against the IE protein only and was MHC 1 A3 haplotype restricted. Equine MHC 1

tetramers, comprising four identical MHC peptides each bearing a biotin and linked to a fluorophore-tagged streptavidin molecule, have the potential to allow identification of MHC class I alleles restricting CTL responses to EHV-1 and identification of EHV-1-derived peptides eliciting CTL responses (Ellis, Compton, UK). This technology will require further development work as there is considerable MHC polymorphism and although the Thoroughbred population has a relatively small number of serologically defined MHC 1 haplotypes there may be as many as 30–40 different alleles involved. However, equine MHC 1 tetramers have been successfully employed in identifying EIAV proteins that contain CTL epitopes and their MHC restriction and hold great promise for future EHV-1 immunological studies (Ellis, Compton, UK).

5. Immune evasion

The changes that occur in blood and pulmonary leukocyte populations after EHV-1 infection, including the lack of virus antigen expression on the surface of infected leukocytes (van der Meullen, Ghent, Belgium) has led to the recognition that EHV-1, in common with many of the herpesviruses, may be capable of modulating the horse's immune response. Since cytotoxicity against EHV-1 proteins is MHC 1 restricted (Lunn, CO, USA; Kydd, Newmarket, UK) it is not surprising that EHV-1 produces proteins that interfere with MHC 1 presentation of virus antigens as a means of evading the horse's immune response to infection (Ellis, Compton, UK). Whether this *immunomodulation* represents *immunosuppression* is an area that requires further investigation (Babiuk, Saskatchewan, Canada). EHV-1 causes specific down-regulation of MHC 1 on the surface of infected cells, shown by FACS analysis of NBL-6 cells infected in vitro (Rappocciolo et al., 2003). MHC 1 down-regulation is mediated by the IE, or possibly E, proteins of EHV-1 and is incomplete, possibly representing an allele or locus specific mechanism involving redirection of class I heavy chains to endolysosomes for degradation as in MCMV or rapid endocytosis from the cell surface as in HHV-8. Preliminary characterisation of which EHV-1 genes are responsible for MHC 1 down regulation has been carried out using NBL6 cells

transfected with a variety of EHV-1 genes (1, 2, 11, 35, 35.5, 63, 65, 69, 71, 75). Of these, only five (1, 2, 35, 63, 69) were expressed and there was decreased MHC-1 expression on the surface of ORF 1 transfected cells.

Whilst a large amount of experimental data has been generated from experimental infection studies using horses, there has been much effort within the international scientific community to develop laboratory animal infection models to reduce the need for experimental horses. Millar Halley (Sydney, Australia) reviewed the use of laboratory animal models for EHV-1, which has included mice, hamsters, guinea pigs, rabbits and kittens. The mouse intranasal infection model (Awan et al., 1991, 1995) has many similarities to natural infection of horses, although both mouse- and virus-strain influence the outcome of infection. In contrast to the horse there is no mouse–mouse transmission of virus and the differences between the equine and murine immune systems raise questions over the modelling of immune parameters against EHV-1 infection in mice. Intranasal infection of mice causes pulmonary changes consisting of bronchiolar epithelial infection with intranuclear inclusions, inflammatory exudate and perivascular mononuclear cell infiltrates leading to the suggestion that the respiratory aspect of the mouse model (as opposed to immunology, abortion and latency aspects) is the most meaningful. The immunogenicity of a variety of EHV-1 antigens, including gB, gC, gH/L, gp2, IE and UL5, delivered as baculovirus expressed proteins or DNA, have been tested in mice using respiratory parameters to compare pathogenicity.

6. Vaccination

The history of EHV-1 vaccination was reviewed by Hugh Townsend (Saskatchewan, Canada). The first EHV-1 vaccine was a live hamster-adapted strain used in Kentucky in 1961 (Doll, 1961) for a 'planned infection programme'. Horses were protected for 3 months against respiratory disease (Doll and Bryans, 1963) and field data suggested a reduction in abortions, although it was suspected that vaccination caused abortion in some mares. Observations on the frequency of abortion by month of pregnancy led to recommendations that vaccination should be carried out in months 5, 7 and 9.

Bryans and Allen (1982) reported that in 3 years of Pneumobort K use in Kentucky 140/20,223 non-vaccinated mares aborted (0.69%) compared to 14/6806 vaccinated mares (0.18%). Although these data suggested that abortion was, in fact, a rather rare event, they provided the first evidence that vaccination may decrease abortion. This observation was not supported, however, by an experimental challenge study (Burrows et al., 1984). Six out of fifteen Pneumobort K-vaccinated mares aborted compared to 2/7 non-vaccinated mares and there was no difference in viremia between the two groups. There is a paucity of data on the efficacy of EHV-1 vaccination from randomised, controlled studies, although a study of this type has been carried out with a commercial inactivated combined EHV-1/-4 vaccine (Heldens et al., 2001). For respiratory disease there was no difference between vaccinates and non vaccinates, although vaccination did decrease nasal shedding but not viremia. For abortion, vaccination did not influence viremia but did appear to decrease abortion (4/5 vaccinates foaled whereas 4/5 non vaccinates aborted). However, these mares had existing CTLp, which may have influenced the outcome of challenge (Kydd, Newmarket, UK). A spontaneous temperature sensitive EHV-1 mutant has been tested in a randomised controlled respiratory and abortion challenge study (Patel et al., 2003a,b). Vaccination decreased respiratory clinical signs and pyrexia, decreased viremia but did not decrease nasal shedding of virus. In the abortion study, viremia was reduced and fewer vaccinated mares aborted. The current vaccine situation is that there are 10 killed commercial vaccines available (8 in the USA and 2 in Europe) and 2 live vaccines (1 in the USA and 1 in Europe). It would appear that these vaccines offer reasonable protection against respiratory disease, although there are few data to support this. The effect of vaccination on abortion in the field is less clear because the rarity of field abortions makes these studies difficult. A reliance is placed on experimental studies but there is a need for properly randomised, blindly-controlled studies to produce reliable data. Further complications are a lack of standardised clinical scoring systems (Minke, Lyon, France) and the question of whether it is desirable to use naïve mares for these studies (Davis-Poynter, Newmarket, UK).

Several presentations reported experiences with experimental and field EHV-1 vaccination strategies. The virus was first isolated in Australia in 1975 with the first abortion storm in 1977 (Foote, Sydney, Australia). Since then sporadic abortions have continued but abortion storms are uncommon. A 'silent' cycle of infection in mares and foals on a large Hunter Valley stud farm has been identified with mare to foal infection and then spread within foals both before and after weaning (Foote et al., 2003, 2004). The effect of vaccination with a commercial killed EHV-1/-4 vaccine was followed in 237 mares and their foals using the gG-specific ELISA and a EHV-1/-4 gC-specific PCR to identify seroconversion and virus shedding. Vaccination of mares had little effect on the silent infection cycle and foals still became infected with both EHV-1 and EHV-4. Overall, small numbers of foals shed infectious EHV-1 and seroconverted (16/237) but more foals (44/237) shed EHV-4 and seroconverted. One-third of the EHV-1 positive foals (5/16) were from vaccinated mares and three quarters of the EHV-4 positive foals (33/44) were from vaccinated mares. Experimental studies have been carried out in mice and horses using baculovirus-expressed gB delivered as DNA prime and protein boost (Foote, Sydney, Australia). In mice vaccination with DNA alone did not induce satisfactory antibody responses whereas protein vaccination, with or without DNA vaccination, did induce a satisfactory response. In an abortion challenge study using 15 mares and their foals, vaccination decreased virus shedding in both mares and their foals suggesting that vaccination-induced antibody in foals was more effective at preventing foal disease than colostral antibody. A number of recombinant vaccines have been used in horses to address the incomplete protection induced by inactivated vaccines (Minke, Lyon, France). Vaccination has been carried out in horses using a canarypox virus expressing gB, gC, and gD co-driven by the HCMV IE promoter and also a DNA (plasmid) vector derived from this virus expressing the same glycoproteins. The effect of different adjuvants (aluminium phosphate, carbopol and GMCSF plus the rabies adjuvant DMIE-DOPE) has been evaluated for both recombinant canarypox virus and DNA vaccination. The carbopol adjuvant appeared optimal for canarypox virus vaccination whereas aluminium phosphate

provided the best antibody responses for DNA vaccination; as had been noted by other investigators (Whalley and Foote, Sydney, Australia), DNA vaccination without adjuvant did not induce a measurable antibody response. Overall, both canarypox virus and DNA vaccination produced similar results with reduced nasal shedding of virus but not viremia. It appears from these studies that it is relatively easy to induce VN antibody and reduce nasal shedding of virus but controlling viremia remains a difficult goal. Paul Lunn (CO, USA) reported the effect of mucosal DNA vaccination on antibody and CTL responses in ponies using a plasmid encoding five EHV-1 ORFs: IE, UL5 (E), gB, gC and gD. Vaccination did not appear to increase VN antibody titres or CTL responses whereas both increased after challenge. Vaccination trials with gE and gI deletion mutants delivered by IM injection have been carried out in colostrum-deprived foals (Matsumura, Tochigi, Japan) but without any obvious effect on virus shedding or viremia.

7. Conclusions

Great advances have been made in understanding the equine immune response to EHV-1 (Horohov, KY, USA). We now have a good understanding of the humoral immune response and better understanding of the cellular immune response, particular with regard to CTL responses. More work is needed to elucidate the role of CD4+ cells (Kydd, Newmarket, UK) and the reliability of IFN gamma as a surrogate marker of CTL activity (Breathnach et al., 2005; Paillot et al., 2005) (Antzcak, Cornell, USA). We finally have some insight into the virus antigens that serve as CTL targets although the complexity of the interaction with MHC haplotype is a reminder that this is no simple quest. The correlates of protective immunity for EHV-1 are still unclear (Horohov, KY, USA) and a further consideration is how such protective responses could be effectively generated if first exposure to EHV antigens may shape the future response and a multi-component response is almost certainly required.

A number of clinical, virological and immunological research priorities were identified (Antzcak, New York, USA).

1. Clinical priorities: Internationally standardized and optimized diagnostic protocols need to be determined, especially with regard to respiratory tract sampling sites and sampling methods, virus isolation, type-specific ELISAs and PCR methods. EHV-1 control programmes would be greatly aided by the establishment of an international disease-reporting scheme. The influenza-virus reporting scheme and the Flu-Net resource have been extremely useful disease control tools and an international collating centre for EHV-1 isolates would dramatically speed EHV-1 surveillance and research. The codes of practice for controlling EHV infections should be revised to include guidelines on the management of outbreaks of neurological disease.
2. Virological priorities: BAC technology coupled with targeted or random (transposon) mutagenesis offer unprecedented opportunities to unravel virus gene function and may prove more timely than extensive genome sequencing of different strains, although the latter approach may continue to prove useful for virulence determinant identification and molecular epidemiology (David-Poynter, Newmarket, UK).
3. Immunological priorities: It is now clear that the individual horse's MHC haplotype plays a major role in recognition of virus antigens and driving the cellular immune response. Further dissection of the role of MHC haplotype in the immune response to EHV is required together consideration of other immunological systems. It is also important that horses for experimental challenge studies have known haplotypes to ensure validity of data. Some progress has been made in identifying virus antigens that serve as epitopes for cytotoxic responses. This work needs to be extended to identify all the EHV-1 proteins recognised by CTL. Internationally agreed standards for the design of vaccine trials must be agreed as a priority. At present there are differences between registration and legislative requirements in Europe and the USA; the immunological and virological status of horses used in trials is not standardised, nor is there agreement on the desirability of EHV-naïve animals; international consensus is required on the issue of optimum group size and whether trials should be large enough to allow randomization.

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