Proceedings of a Workshop on

EQUINE IMMUNOLOGY IN 2001

24th–28th January 2001
Santa Fe, New Mexico

Editors: D. P. Lunn and J. F. Wade
Proceedings of a Workshop on

EQUINE IMMUNOLOGY IN 2001

24th–28th January 2001
Santa Fe, New Mexico

Editors: D. P. Lunn and J. F. Wade
CONTENTS

FOREWORD .......................................................................................................................................Page vi

SESSION I: IMMUNITY & INFECTION

Immune control of equine infectious anaemia virus
T. C. McGuire, S. R. Leib, R. H. Mealey, D. G. Fraser, S. L. Ridgely and D. J. Prieur ..................Page 3

Overview of T cell cytokine research in the horse
D. W. Horohov ......................................................................................................................................Page 7

Rhodococcus equi, a paradigm for equine immunity to intracellular bacteria
S. Giguère ................................................................................................................................................Page 11

Equine herpesvirus-1: immunity in lytic and latent infections
J. Slater ............................................................................................................................................Page 14

Strongyles large and small: immunity
T. R. Klei ........................................................................................................................................Page 17

Mucosal immunity: an opportunity to prime without prejudice
D. Hannant........................................................................................................................................Page 19

Mucosal antigen delivery in horses
J. F. Timoney and A. S. Sheoran ............................................................ Page 21

Regulation of mucosal immune responses
G. Sobboll, D. W. Horohov, C. W. Olsen and D. P. Lunn ...............................................................Page 23

The induction of equine herpesvirus-specific antibodies in the upper respiratory tract of the horse

Immunogenetics defined
D. F. Antczak .......................................................................................................................................Page 29

Organisation of the equine immunoglobulin constant region genes
B. Wagner ........................................................................................................................................Page 30

Equine MAC regulation of resistance and immune bias
E. Marti ................................................................................................................................................Page 33

A syndrome of anaemia, immunodeficiency and peripheral ganglionopathy in Fell pony foals
M. A. Holmes, S. F. E. Scoles, A. Holliman and P. D. F. May .......................................................Page 35
SESSION II: NEW TECHNOLOGIES IN EQUINE IMMUNITY

Monoclonal antibodies: what’s next?
D. P. Lunn .....................................................................................................................................Page 41

Detection of interferon-γ producing equine T lymphocytes by flow cytometry:
pulmonary responses to Rhodococcus equi challenge

II-4 induced CD23 (FCERII) upregulation in equine peripheral blood mononuclear cells and
alveolar macrophages
J. L. Watson, K. A. Jackson and J. L. Stott ........................................................................................Page 44

Oponisation by complement C3 and IgG as measured by a flow cytometric immunoassay
G. Gröndahl .......................................................................................................................................Page 46

Flow cytometric characterisation of neutrophil phagocytosis and oxidative burst activity
S. L. Raidal ........................................................................................................................................Page 49

RT-PCR detection of equine cytokines
D. W. Horohov ...................................................................................................................................Page 54

Equine cytokines and associated reagents
L. Nicolson, L. McMonagle, S. Taylor, C. Hopkins, L. Sanders, H. van Kuilekom, N. Scholts,
D. Argyle, D. Onions and V. Schijns ...............................................................................................Page 57

Quantitation of equine cytokine mRNA expression by RT-PCR
S. Giguère .........................................................................................................................................Page 59

Type I interferon and interleukin-6 in nasal secretions and serum from ponies infected
with equine influenza A2 (H3N8)
E. Wattrang, D. M. Jessett, P. Yates, L. Fuxler and D. Hannant .......................................................Page 62

Genomic exploration of orthopaedic infection using cDNA microarrays
E. M. Santschi, A. Rink and C. W. Beattie ........................................................................................Page 64

The power of limiting dilution analysis
J. H. Kydd, E. Wattrang, G. P. Allen, T. O’Neill and D. Hannant .....................................................Page 65

Flow cytometric techniques in clinical investigation

Simultaneous analysis of phagocytosis and oxidative burst activity of equine phagocytes by
flow cytometry

SESSION III: INFLAMMATION

Chronic airway disease in the horse
N. E. Robinson ..................................................................................................................................Page 73

Variation of non-infectious airway disease phenotype with age
L. Viel ................................................................................................................................................Page 77

Biology of airway neutrophils
B. McGorum and T. Brazil ................................................................................................................Page 79

Cytokine immunoregulatory elements in RAO
J. P. Lavoie and D. W. Horohov .......................................................................................................Page 82

IgG subtypes and clues to the immunopathology of recurrent airway obstruction (heaves)
D. M. Ainsworth, J. A. Appleton, D. F. Antczak, M. A. Santiago and G. A. Aviza .............................Page 84
Effects of dust and endotoxin exposures on lung function and airway cytology of horses
L. L. Couëtil, M. A. Hunt and F. S. Rosenthal .................................................................Page 86

Septicaemia and endotoxaemia in horses
J. N. Moore .................................................................................................................. Page 87

Pathophysiology of endothelin-1 in equine acute laminitis

Immunomodulation of events in sepsis: role of P-selectin/P-selectin ligand system
B. J. Darien................................................................................................................... Page 91

Joint disease in the horse
C. W. McIlwraith..........................................................................................................Page 94

Chondrocytic response to joint inflammation and corticosteroids
J. MacLeod....................................................................................................................Page 97

Regulation of inducible nitric oxide expression and activity in equine chondrocytes
J. T. Tung, P. J. Venta and J. P. Caron .........................................................................Page 99

LIST OF PARTICIPANTS..............................................................................................Page 102

AUTHOR INDEX.........................................................................................................Page 103
The Havemeyer Foundation Workshop on Equine Immunology in 2001 was convened in order to bring together a broad range of equine scientists with a common interest in immunological studies, both basic and applied. The goal of the organisers was to ensure that the latest and greatest were presented, and that innovative scientists working in different disciplines would have the opportunity to learn about new tools and establish new collaborations. We were delighted by the quality of the presentations and the meeting in general, the strength of which is well documented by the outstanding papers in this monograph. The standard of research and results presented illustrates clearly the ongoing progress in the field of equine immunology, and promises much for the future. It is our hope and expectation that the relationships and collaborations established by this workshop will foster this progress.

We are extremely grateful to the Havemeyer Foundation, and to Mr Gene Pranzo, for providing the major sponsorship for the meeting. In addition we would like to thank the Veterinary Immunology Committee of the International Union of Immunological Societies, Heska, Bayer and Intervet for additional support. We are also extremely grateful to Mrs Jan Wade and R&W Publications for managing the organisation of the meeting and for the preparation of this monograph.

We hope the reader finds much of value in this publication, and feel that it is a fitting testament to the breadth and strength of equine immunological science in this new millennium.

D. Paul Lunn
David W. Horohov
Doug F. Antczak
1981  First International Workshop on Lymphocyte Alloantigens of the Horse  
October - New York City, USA  
Organiser: Dr D. F. Antczak

1982  Second International Workshop on Lymphocyte Alloantigens of the Horse  
October - Cornell University, Ithaca, New York, USA  
Organiser: Dr D. F. Antczak

1983  Third International Workshop on Lymphocyte Alloantigens of the Horse  
April - New Bolton Center, University of Pennsylvania, USA  
Organiser: Dr D. F. Antczak

1984  First International Symposium on Equine Embryo Transfer  
October - Cornell University, Ithaca, New York, USA  
Organisers: Drs D. F. Antczak and W. R. Allen

1985  Fourth International Workshop on Lymphocyte Alloantigens of the Horse  
October - University of Kentucky, USA  
Organisers: Drs D. F. Antczak and E. Bailey

1986  Workshop on Corynebacterium equi Pneumonia of Foals  
July - University of Guelph, Canada  
Organiser: Dr J. F. Prescott

1987  Fifth International Workshop on Lymphocyte Alloantigens of the Horse  
October - Louisiana State University, USA  
Organisers: Drs D. F. Antczak and J. McClure

1989  Second International Symposium on Equine Embryo Transfer  
February - Banff, Alberta, Canada  
Organisers: Drs D. F. Antczak and W. R. Allen

1990  International Workshop on Equine Sarcoids  
April - Interlaken, Switzerland  
Organisers: Dr D. F. Antczak and Professor S. Lazary

1992  Workshop on Equine Neonatal Medicine  
January - Naples, Florida  
Organisers: Drs D. F. Antczak and P. D. Rossdale

Third International Symposium on Equine Embryo Transfer  
February - Buenos Aires, Argentina  
1995

**Equine Perinatology**
July - Cambridge, England
*Organiser: Dr P. D. Rossdale*

**Second International Equine Leucocyte Antigen Workshop**
July - Lake Tahoe, California, USA
*Organisers: Drs D. F. Antczak, P. Lunn and M. Holmes*

**First International Workshop on Equine Gene Mapping**
October - Lexington, Kentucky, USA
*Organisers: Drs D. F. Antczak and E. Bailey*

**Erection and Ejaculation in the Human Male and Stallion: A Comparative Study**
October - Mount Joy, Pennsylvania, USA
*Organiser: Dr S. M. McDonnell*

**Bone Remodelling Workshop**
October - Concord, Massachusetts, USA
*Organiser: Dr H. Seeherman*

1997

**Second International Workshop on Equine Gene Mapping**
October - San Diego, California, USA
*Organisers: Drs D. F. Antczak and E. Bailey*

**Maternal Recognition of Pregnancy in the Mare**
January - Dominican Republic
*Organisers: Drs W. R. Allen and T. A. E. Stout*

**Uterine Clearance**
March - Gainesville, Florida, USA
*Organiser: Dr M. M. LeBlanc*

**Trophoblast Differentiation**
September - Edinburgh, Scotland
*Organisers: Drs D. F. Antczak and F. Stewart*

1998

**Third International Genome Workshop**
January - San Diego, California, USA
*Organisers: Drs D. F. Antczak and E. Bailey*

**Third International Workshop on Perinatology: Genesis and Post Natal Consequences of Abnormal Intrauterine Developments: Comparative Aspects**
February - Sydney, Australia
*Organiser: Dr P. D. Rossdale*

**Horse Genomics and the Genetic Factors Affecting Race Horse Performance**
March - Banbury Center, Cold Spring Harbor, New York, USA
*Organisers: Drs D. F. Antczak, E. Bailey and J. Witkowski*
Allergic Diseases of the Horse  
April - Lipica, Slovenia  
Organisers: Drs D. F. Antczak, S. Lazary and E. Marti

Equine Placentitis Workshop  
October - Lexington, Kentucky, USA  
Organisers: Drs D. F. Antczak, W. R. Allen and W. Zent

Septicemia II Workshop  
November - Boston, Massachusetts, USA  
Organiser: Dr M. R. Paradis

1999  
Equine Genome Project  
January - San Diego, California, USA  
Organisers: Drs D. F. Antczak and E. Bailey

Third International Equine Genome Workshop  
June - Uppsala, Sweden  
Organisers: Drs D. F. Antczak, E. Bailey and K. Sandberg

Fourth International Meeting of OIE and WHO Experts on Control of Equine Influenza  
August - Miami, Florida, USA  
Organiser: Dr J. Mumford

European Equine Gamete Workshop  
September - Lopuszna, Poland  
Organisers: Drs W. R. Allen and M. Tischner

Fetomaternal Control of Pregnancy  
November - Barbados, West Indies  
Organisers: Drs T. Stout and W. R. Allen

2000  
Equine Genome Project  
January - San Diego, California, USA  
Organisers: Drs D. F. Antczak and E. Bailey

Uterine Infections in Mares and Women: A Comparative Study  
March - Naples, Florida, USA  
Organiser: Dr M. M. LeBlanc

5th International Symposium on Equine Embryo Transfer  
Saari, Finland  
Organiser: Dr T. Katila

2001  
USDA International Plant & Animal Genome Conference  
San Diego, California

Equine Immunology in 2001  
Sanata Fe, New Mexico  
Organiser: Dr D. P. Lunn
HAVEMEYER MONOGRAPH SERIES

The following are monographs available to date at a cost of £9.95 each.

**Series No 1**
**PROCEEDINGS OF THE FIRST MEETING OF THE EUROPEAN EQUINE GAMETE GROUP (EEGG)**
Editors: W. R. Allen and J. F. Wade
5th–8th September 1999
Lopuszna, Poland

**Series No 2**
**PROCEEDINGS OF A WORKSHOP ON FETOMATERNAL CONTROL OF PREGNANCY**
Editors: T. A. E. Stout and J. F. Wade
14th–16th November 1999
Barbados, West Indies

**Series No 3**
**PROCEEDINGS OF THE 5TH INTERNATIONAL SYMPOSIUM ON EQUINE EMBRYO TRANSFER**
Editors: T. Katila and J. F. Wade
6th–9th July 2000
Saari, Finland

**Series No 4**
**PROCEEDINGS OF A WORKSHOP ON EQUINE IMMUNOLOGY IN 2001**
Editors: D. P. Lunn and J. F. Wade
24th–28th January 2001
Santa Fe, New Mexico

If you wish to order copies, please contact R & W Publications Ltd, Suites 3 & 4, 8 Kings Court, Willie Snaith Road, Newmarket, Suffolk CB8 7SG, UK, Tel: +44 1638 667600, Fax: +44 1638 667229, e-mail: rw.publications@btinternet.com.
SESSION I:

Immunity & infection

Chairman: David Horohov
Horses with equine infectious anaemia virus (EIAV) are infected for life, but control of virus replication occurs following the viraemic episodes that usually happen during the first year of infection. Later, during the carrier state when there are no clinical signs, the virus is clearly more tightly controlled and virus replication is difficult to detect. An adaptive immune response is required for the control of initial viraemia. This was demonstrated by the failure of Arabian foals with severe combined immunodeficiency (SCID) and lacking functional T and B lymphocytes (McGuire et al. 1974) to control viraemia after infection with EIAV (Perryman et al. 1988). Non-SCID foals controlled viraemia after similar infection. In addition, adoptive transfer of lymphocytes from an EIAV-infected horse to a SCID foal matched at the A locus of MHC class I resulted in control of viraemia following challenge (Mealey et al. 2001).

At least 2 immunological mechanisms for EIAV control have been identified, neutralising antibody and cytotoxic T lymphocytes (CTL). Neutralising antibody is effective when it is present to homologous virus. However, in infected horses, continual antigenic variation of recognised epitopes limits its effectiveness. In addition, the late appearance of neutralising antibody after infection, or after the appearance of variants, further constrains the effectiveness of neutralising antibody in EIAV control (Montelaro et al. 1993). Another possible protective mechanism involving antibody is antibody dependent cellular cytotoxicity (ADCC). However, ADCC against EIAV-infected target cells could not be demonstrated with sera from infected horses, whereas ADCC against equine herpesvirus-1 (EHV-1)-infected target cells could be demonstrated with sera from horses vaccinated against EHV-1 (Tschetter et al. 1997). Thus, the absence of antibodies which cause ADCC, and the late appearance of neutralising antibody as well as variation of neutralising antibody sensitive epitopes, dictates additional focus on CTL.

Our research hypothesis is that class I-restricted, EIAV-specific CD8+ CTL control EIAV infection. Effector CTL that do not require in vitro stimulation and kill EIAV-infected target cells are present in peripheral blood mononuclear cells (PBMC) within a few days of initial viraemia and for approximately 3 months after infection (McGuire et al. 1994). This observation is consistent with these cells being responsible for control of the initial viraemia. Later in infection, in vitro stimulation with antigen is required to demonstrate MHC class I-restricted, EIAV-specific CD8+ memory CTL (McGuire et al. 1997; Hammond et al. 1997). In addition, EIAV-specific, MHC class II-restricted CD4+ memory CTL have been demonstrated in EIAV-infected horses (Hammond et al. 1997). The importance of these class II-restricted CTL is not known. Many of our studies use equine kidney (EK) cells which express MHC class I molecules, but not class II molecules (McGuire et al. 1997). Therefore, the only CTL activity that can be measured in this system is that mediated by CD8+ T lymphocytes. In order to have autologous and MHC class I-mismatched EK cells for targets in CTL assays, kidney biopsies are taken before EIAV infection and the cells are expanded and frozen for later use. Other investigators have effectively used stimulated horse T lymphocytes as target cells in CTL assays (Allen et al. 1995; O’Neill et al. 1999).

To determine if memory CTL were present in EIAV carriers and in numbers that could account for virus control, the CTLm frequency in PBMC
from 5 inapparent carriers infected for 22 to 50 months was determined by limiting dilution analysis (McGuire et al. 1997). Limiting dilution analysis has also been used to determine the frequency of CTLm to EHV-1 in horses (O’Neill et al. 1999). PBMC from EIAV carrier horses were diluted, stimulated and tested on EK cell targets infected with EIAV and recombinant vaccinia viruses expressing EIAV Env or Gag/Pr proteins. All 5 carriers had CTLm to EIAV-infected targets; 3 had CTLm to targets expressing either Env or Gag/Pr proteins, one had CTLm to Env only and one had CTLm to Gag/Pr only. The CTLm frequency range was 60–468 per million PBMC to EIAV-infected targets, 4–286 to Env, and 25–190 to Gag/Pr expressing targets (McGuire et al. 1997). In summary, the mean frequency of memory CTL to EIAV-infected target cells in PBMC from carrier horses is 293/million (1/3,413) when measured by limiting dilution analysis, which underestimates frequency. This number of memory CTL is consistent with these cells being responsible for EIAV control in the carrier state.

Because proteins and epitopes recognised by predominant memory CTL from inapparent carriers may be useful for inducing protective immune responses in naïve horses, experiments were undertaken using these memory CTL to identify the EIAV proteins and epitopes for further evaluation in immunisation experiments. To identify the EIAV proteins with epitopes recognised by class I-restricted CD8+ CTL from the greatest number of carrier horses, retroviral vectors expressing the different EIAV proteins were made (Lonning et al. 1999a). These retroviral vectors were used to transduce EK cells for targets in CTL assays (McGuire et al. 2000). The proteins recognised by memory CTL from the most carrier horses was Gag p15 (by CTL from 100% of 7 infected horses), p26 (86%), SU and the middle third of Pol protein (each by 43%), TM (29%), and S2 (14%). These results identify the proteins recognised by the CTL present during times of virus control and justify experiments to determine their role.

As most carrier horses had memory CTL that recognised Gag matrix (p15) and capsid (p26) proteins, with no killing of target cells expressing p11 and p9, overlapping synthetic peptides of 16–25 amino acids were used to identify p15 and p26 epitopes (Zhang et al. 1998). Peptides were identified containing at least 12 Gag CTL epitopes recognised by virus-stimulated PBMC from 6 long-term EIAV-infected horses with varying class I haplotypes. Each of the 6 horses had CTL recognising at least one Gag epitope, and CTL from one horse recognised at least 8 different Gag epitopes. None of the identified peptides were recognised by CTL from all 6 horses. Two nonamer peptide epitopes were defined from Gag p26; one (18a) was restricted by class I equine leucocyte alloantigen (ELA) -A5.1 and the other (28b-1) by ELA-A9 molecules (Zhang et al. 1998). A 10 nM concentration of peptide 18a was required to sensitise EK target cells for CTL killing whereas 28b-1 required 1 nM. The results demonstrated that diverse CTL responses against Gag epitopes were generated in EIAV-infected carrier horses and indicated that ELA-A class I molecules were responsible for the diversity of CTL epitopes recognised. Further work demonstrated that neither epitope 18a or 28b-1 were conserved among EIAV strains indicating that antigenic variation of these epitopes could occur (Zhang et al. 1999).

To determine the in vivo role of CD8+ CTL, we are inducing CTL in horses expressing class I ELA-A1 molecules by immunisation with peptides containing mapped A1-restricted CTL epitopes. When CTL that will kill EIAV-infected target cells are detected in numbers equal to or higher than the number of CTLm detected in carrier horses, then the immunised horses will be challenged with EIAV and the protective response mediated by the CTL evaluated. This approach is being taken to lessen the variables associated with inducing CTL responses in outbred horses to protein antigens. To this end, a class I ELA-A1-restricted EIAV peptide has been defined by initial mapping with retroviral vector-transduced target cells expressing all different EIAV proteins including portions of the Env proteins (McGuire et al. 2000). CTL assays with these targets identified an epitope in Env construct 1 (Fig 1). The protein expressed by Env 1 was expressed in 3 parts by 3 new vectors (a,b,c) (Fig 2) and Env T1c was found to contain the A1-restricted epitope (Fig 3). Six overlapping synthetic peptides covering the protein of Env T1c were constructed and used for further CTL epitope mapping and peptide 1 was found to contain the epitope (Fig 3). Further mapping to define the minimal CTL epitope is being done. The class I molecules from an A1 horse have been cloned and expressed in order to identify the gene expressing the A1 molecule. In addition, EIAV peptides recognised by CD4+ Th1
lymphocytes of carrier horses with varying MHC class II haplotypes have been identified to evaluate their potential to enhance CTL responses (15,16). Finally, if induction of CTL in horses is required for protection against EIAV or any other disease, immunization strategies must induce CTL to epitopes in various proteins, in horses with different MHC class I haplotypes.

REFERENCES


OVERVIEW OF T CELL CYTOKINE RESEARCH IN THE HORSE

D. W. Horohov

Department of Pathobiological Sciences, Louisiana State University, School of Veterinary Medicine, Baton Rouge, Louisiana 70803, USA

Cytokines are small hormone-like proteins that influence the functions of various cells. A vast number of cytokines have been described that influence lymphocyte function. Work in murine models has made it clear that the induction of protective resistance or the exacerbation of disease is often dependent upon the pattern of cytokine genes expressed during an immune response (Mosmann and Coffman 1989; Urban et al. 1996; Lohoff et al. 1998). Although research into the role of cytokines in equine immunity and disease is in its infancy, considerable advances have been made. The horse presents a number of scenarios that lend themselves to the study of cytokine interactions in either disease progression or prevention. The emphasis of the earliest work in these areas was on the characterisation of the cytokine response itself, first through the use of conventional bioassays (Stott and Osburn 1988; Morris et al. 1990; May et al. 1990; Morris et al. 1992) and later, with the growing availability of recombinant cytokines and monoclonal antibodies, through immunoassays (MacKay and Socher 1992; Franchini et al. 1998) and molecular analyses (Giguère and Prescott 1999; Swiderski et al. 1999c; Leutenegger et al. 1999). Although relatively few groups have been involved in these efforts, the tools of modern biotechnology have helped to accelerate the rate of advancement. Thus, the cloning, sequencing and expression of a number of equine cytokines have been accomplished (Table 1). The availability of these reagents will help lead to a better understanding of the equine immune system and, perhaps, of the immunological basis of a number of important equine diseases.

Recent work has focused on the patterns of cytokine expression in various disease processes in the horse and the role that specific cytokines may play in either protection or pathology. These studies are modelled in part on the early observations made in the mouse that T lymphocytes could be divided into different subpopulations based on their pattern of cytokine expression and these patterns of cytokine expression could be associated with either protective or pathological responses to various parasitic agents (Urban et al. 1996; Mosmann and Coffman 1989; Lohoff et al. 1998). Thus, so-called Th1 cells produced those cytokines, notably interferon (IFN)-γ and interleukin (IL)-2, which generated protective immune responses to intracellular parasites (Lohoff et al. 1998). By contrast, Th2 cells produced those cytokines, IL-4 and IL-5, which played a central role in immunity to intestinal helminths and other multicellular parasites (Urban et al. 1996). In addition to these protective responses, it was also shown that both subsets could be associated with immunopathological responses. Many forms of autoimmune disease appear to be associated with the induction of a Th1 response to self-antigens.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Genebank accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>D42146, U92481</td>
</tr>
<tr>
<td>IL-2</td>
<td>L06009, X69393</td>
</tr>
<tr>
<td>IL-4</td>
<td>L06010</td>
</tr>
<tr>
<td>IL-5</td>
<td>U91947</td>
</tr>
<tr>
<td>IL-6</td>
<td>U64794, AF005227</td>
</tr>
<tr>
<td>IL-8</td>
<td>AF062377</td>
</tr>
<tr>
<td>IL-10</td>
<td>U38200</td>
</tr>
<tr>
<td>IL-12</td>
<td>Y11129, Y11130</td>
</tr>
<tr>
<td>IL-18</td>
<td>Y11131</td>
</tr>
<tr>
<td>IFN-α</td>
<td>M14540</td>
</tr>
<tr>
<td>IFN-8</td>
<td>M14546</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>D28520, U04050</td>
</tr>
<tr>
<td>TNF-α</td>
<td>M64087</td>
</tr>
</tbody>
</table>
(O’Garra et al. 1997). Likewise, allergies are associated with the induction of a Th2 response (Umetsu and DeKruyff 1997). Indeed atopic individuals have a propensity to mount Th2 responses to most foreign antigens (Del Prete 1992). While similar patterns of cytokine expression have been observed in other species, their existence in the horse was unknown. Nevertheless there are several clinical situations in which it appears likely that Th1 or Th2 cells could play a central role in either the protective or pathological response in horses.

Horses affected with equine recurrent uveitis (ERU) experience painful bouts of ocular inflammation that can ultimately lead to blindness in the affected eye (Schwink 1992). Although the underlying cause of this condition is unknown, data support the hypothesis that this disease is immunological in nature (Romeike et al. 1998). Analysis of mRNA collected from the eyes of ERU horses demonstrated the presence of messenger (m)RNA for the Th1 cytokines IL-2 and IFN-γ (Gilger et al. 1999). Furthermore, treatment of affected horses with corticosteroid implants resulted in both a reduction in cytokine mRNA levels and an improvement of the clinical condition (Gilger et al. 2000). Direct cause has yet to be established but these results are consistent with the notion that ERU is the result of an ongoing Th1 immune response in the affected eye.

A Th1 response was also associated with protective immune responses to equine influenza virus infection in ponies. This response, primarily associated with the respiratory lymph nodes of infected ponies, contrasted with the response in ponies given a commercial vaccine (Fig 1). Indeed the latter response was characterised by the lack of IFN-γ production and elevated levels of the Th2 cytokine IL-4, presumably as a result of the use of alum in the commercial product (Grun and Maurer 1989). Given that horses infected with equine influenza virus exhibit longer term protection than vaccinates (Hannant et al. 1988), these results are consistent with murine models demonstrating that Th1 cells play a central role in protection against influenza viruses while Th2 cells are non-protective (Graham et al. 1994).

Immunity to the intestinal parasite Strongylus vulgaris is associated with production of the Th2 cytokines IL-4 and IL-5 in both peripheral blood mononuclear cells and colonic lymph nodes (Swiderski et al. 1999a,b). This is consistent with the in vivo observation that protection is associated with the induction of an anamnestic eosinophilia (Dennis et al. 1993) and that IL-5 plays a key role in regulating this response (Dent et al. 1990). By contrast, intramuscular vaccination of ponies with soluble adult worm antigens in a Ribi adjuvant resulted in both disease exacerbation following challenge (Monahan et al. 1995) and the concomitant induction of a Th1 cytokine response (Fig 2). By contrast, oral immunisation with irradiated larvae is associated with a protective Th2 immune response (Fig 2).

Lastly, lymphocytes obtained from bronchoalveolar lavage samples from horses affected with summer pasture-associated obstructive pulmonary disease (SPAOPD), a recurrent airway obstructive disease similar to chronic obstructive pulmonary disease (COPD) (Seahorn et al. 1996), produced significantly elevated levels of IL-4 mRNA. Interestingly mRNA for IL-5 was not detected, suggesting that this is a modified Th2 response (Beadle et al. 2001). Consistent with this interpretation is the
absence of an eosinophilic infiltrate in the lungs of the affected ponies (Seahorn et al. 1996).

In summary, current data support the proposition that horses can mount both Th1 and Th2 immune responses and these can be associated with either protective or pathological responses (Table 2).

TABLE 2: Equine Th1 vs Th2 Paradigm

<table>
<thead>
<tr>
<th>Protection</th>
<th>Pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1</td>
<td>Equine influenza virus</td>
</tr>
<tr>
<td>Th2</td>
<td>Strongyles vulgaris</td>
</tr>
</tbody>
</table>


Rhodococcus equi, a Gram-positive facultative intracellular pathogen, is one of the most important causes of pneumonia in foals aged 1–5 months. Other less common clinical manifestations of R. equi infections in foals include ulcerative enterocolitis, colonic or mesenteric lymphadenopathy, immune-mediated synovitis and uveitis, osteomyelitis and septic arthritis. R. equi is widespread in the environment of stud farms. Unlike most environmental R. equi, isolates from pneumonic foals typically contain an 80–90 kb plasmid encoding a family of 7 closely related virulence-associated proteins designated VapA and VapC through VapH (Takai et al. 2000). Plasmid-cured derivatives of virulent R. equi strains lose their ability to replicate and survive in macrophages, and fail to induce pneumonia in foals, confirming the absolute necessity of the large plasmid for the virulence of R. equi (Giguère et al. 1999a). Cell wall mycolic acid-containing glycolipids may also contribute to virulence of R. equi. Strains with a longer carbon chain mycolic acid are more virulent as determined by lethality and granuloma formation in mice than those with shorter chains (Gotoh et al. 1991). Other unexplored candidates as virulence factors include capsular polysaccharides as well as cholesterol oxidase, choline phosphohydrolase and phospholipase C exoenzymes (‘equi factors’). However, both capsule and exoenzymes are produced by virulent as well as by avirulent strains, suggesting that their contribution to virulence, if any, is insignificant in comparison to plasmid-mediated functions.

The ability of R. equi to persist in, and eventually destroy, alveolar macrophages is the basis of its pathogenicity. Intracellular persistence correlates with the absence of phagosome-lysosome fusion (Hietala and Ardans 1987a; Zink and Yager 1987). Phagocytosis of R. equi by equine macrophages is not associated with a functional respiratory burst (Brumbaugh et al. 1990) and, at least in people, the L-arginine-NO pathway is not required for intracellular killing of this organism (Vullo et al. 1998). Optimal binding of R. equi to mouse macrophages in vitro requires complement and is mediated by Mac-1, a leucocyte complement receptor type 3 (CR3, CD11b/CD18) (Hondalus et al. 1993). Entry of several microorganisms into macrophages after adherence to complement receptors has been shown to allow them to avoid the toxic consequences of the oxidative burst (Baümler and Heffron 1995). Opsonisation of R. equi with specific antibody is associated with increased phagosome-lysosome fusion and significantly enhances killing of R. equi by equine macrophages suggesting that the mechanism of cellular entry can mediate the fate of the bacteria (Hietala and Ardans 1987a). As opposed to macrophages, neutrophils from foals and adult horses are fully bactericidal and killing of R. equi is considerably enhanced by specific opsonising antibody (Hietala and Ardans 1987b; Martens et al. 1988; Takai et al. 1985; Yager et al. 1987).

Immunity to R. equi pneumonia in foals probably depends on both the antibody and cell-mediated components of the immune system but its exact basis remains to be determined. The age of development of R. equi pneumonia coincides with, and may in part be related to, the decline of maternally-derived antibodies (Hietala et al. 1985). However, the strongest evidence for a role of antibody in protection against R. equi is the protective effect of passively transferred anti-R. equi hyperimmune equine plasma (Madigan et al. 11
1991). The mechanisms by which hyperimmune plasma confers protection are not completely understood. The list of possible effector molecules includes antibody and non-specific factors such as fibronectin, complement components, collectins, cytokines and acute phase proteins. Recent studies have focused more specifically on the role of antibody against plasmid-encoded virulence-associated proteins (Vap). First, a monoclonal antibody to VapA and serum from horses immunised with partially purified VapA have opsonising activity (Prescott et al. 1997). Moreover, purified immunoglobulins obtained from horses vaccinated with partially purified VapA protected mice against intraperitoneal challenge with virulent \textit{R. equi} compared with mice administered immunoglobulins from non-immunised horses (Fernandez et al. 1997). More recently, iv administration of purified immunoglobulins obtained from horses immunised with recombinant VapA and VapC to foals was found to reduce the severity of pneumonia following heavy experimental challenge with \textit{R. equi} (Hooper-McGrevy et al. 2001). In the same study, the degree of protection conferred by purified anti-VapA and -VapC immunoglobulins was similar to that provided by hyperimmune plasma.

Because of the facultative intracellular nature of \textit{R. equi}, cell-mediated immune mechanisms are thought to be of major importance in resistance. Almost all knowledge of cell-mediated immunity to \textit{R. equi} infections comes from infection of mice. Deficiencies in the complement component C5, phagocytic cells and NK cells do not impair the pulmonary clearance of virulent \textit{R. equi} (Yager et al. 1991). In contrast, functional T lymphocytes are absolutely required for the clearance of virulent (plasmid and VapA positive) \textit{R. equi} in mice (Kanaly et al. 1996; Madrame et al. 1997; Ross et al. 1996). However, athymic nude mice (lacking functional T lymphocytes) clear plasmid-cured derivatives from their lungs within one week of infection suggesting that, as opposed to virulent organisms, clearance of avirulent plasmid-negative strains in mice does not require functional lymphocytes and depends mainly on innate defence mechanisms (Madrame et al. 1997). The 2 major mechanisms by which T lymphocytes mediate clearance of intracellular pathogens are secretion of cytokines and direct cytotoxicity. Although both CD4\(^+\) (helper) and CD8\(^+\) (cytotoxic) T cells contribute to host defence against \textit{R. equi} in mice, CD4\(^+\) T lymphocytes play the major role and are absolutely required for complete pulmonary clearance (Ross et al. 1996; Kanaly et al. 1993; Nordmann et al. 1992). The mouse CD4\(^+\) Th cells can be divided in 2 subsets based on the cytokines they produce. The Th1 subset produces mainly IFN-\(\gamma\) and IL-2 and is mainly responsible for macrophage activation and cell-mediated immunity. The Th2 subset produces mainly IL-4, IL-5 and IL-10 which mainly promote humoral immunity. Studies in mice have shown that a Th1 response is sufficient to effect pulmonary clearance of \textit{R. equi} whereas a Th2 response is detrimental (Kanaly et al. 1995, 1996). How these findings in mice relate to the foal remains to be determined. Analogy to human immunodeficiency virus-related \textit{R. equi} pneumonia suggests either that foals are immunocompromised in some way or that infection with virulent \textit{R. equi} alters immune response in foals. The cytokine response of foals infected with virulent and avirulent \textit{R. equi} has recently been investigated. Foals infected intrabronchially with a virulence plasmid-containing strain of \textit{R. equi} showed marked reduction in IFN-\(\gamma\) mRNA expression by bronchial lymph node CD4\(^+\) T lymphocytes compared to CD4\(^+\) T cells similarly isolated from foals infected with an avirulent plasmid-cured derivative of the same strain (Giguère et al. 1999b). In addition, IL-10, a cytokine known to downregulate a Th1 response in other species, was only expressed in the lungs of foals infected with the virulent strain (Giguère et al. 1999b). These findings suggest that virulent \textit{R. equi} have an immunomodulating effect important in the pathogenesis of infection.

**REFERENCES**


EQUINE HERPESVIRUS-1: IMMUNITY IN LYTIC AND LATENT INFECTIONS

J. Slater

Department of Clinical Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK

SUMMARY

Understanding of the pathogenesis of lytic and, to a lesser extent, latent EHV-1 infections has improved dramatically. Understanding of the immune response is sufficient to identify that circulating antibody responses alone do not provide a correlate of protective immunity. Preliminary evidence suggests that CTL responses may provide a correlate and CTL responses in the lamina propria and circulation thus require further study. Mucosal immunity is a second area of EHV-1 immunity that requires further investigation.

INTRODUCTION

Equine herpesvirus-1 (EHV-1) establishes invasive infections which result in viraemic dissemination of virus from the respiratory tract to organs throughout the body, notably the uterus and central nervous system (CNS). The virus has a complex lifecycle which involves infection of multiple cell types including epithelial cells, endothelial cells, mononuclear leukocytes and neurones. In addition there are 2 distinct cycles of infection: lytic (‘primary’ or ‘acute’) and latent infection. During lytic infection there is virus replication in infected cells with extensive virus gene transcription and expression of virus antigens on the surface of the infected cell. Lytic infection results in destruction of the infected cell, subsequent release of progeny virions from the cell, infection of adjacent cells and virus shedding from the horse. In contrast, during the latent infection cycle, there is no virus replication; gene transcription is restricted to a single region of the genome and there is no translation of this mRNA. Virus antigens are thus not expressed on the cell surface, the latently-infected cell is not destroyed and progeny virions are not released. During latency, the virus thus resides within latently infected cells in a near-quiescent state. Periodically, however, latent virus undergoes reactivation which may result ultimately in the establishment, once more, of lytic infection in respiratory epithelium, shedding of infectious virus from the respiratory tract and viraemia. The lifecycle of EHV-1 thus presents a major challenge to the immune system because both prevention and termination of infection require an integrated, multi-component immune response to control lytic and latent infection. In recent years, great advances have been made in the understanding of the immune response to EHV-1 and the role of the immune response in pathogenesis. Nonetheless, several key virological and immunological aspects of EHV-1 pathogenesis require further study. The aims of this presentation are to 1) review the current state of knowledge of the immune response to EHV-1 in lytic and latent infections and 2) highlight areas of the immune response that require further study and to consider the tools and techniques currently available to study these areas.

REVIEW OF THE IMMUNE RESPONSE TO EHV-1

Lytic infection

During lytic infection virus gene transcription, and therefore virus antigen expression by infected cells, is tightly regulated into 3 sequential phases: immediate-early, early and late with each phase up-regulating transcription of later phase genes as well as down-regulating earlier phase genes. The first virus gene to be transcribed and the initiator of the lytic infection cycle is the single immediate-early gene, ORF 64. Certain virus antigens have
already been identified as key immunological targets eg ORFs 64 and 5 are potent stimulators of cytotoxic T lymphocyte (CTL) responses and virus glycoproteins gB, gC and gD are targets for neutralising antibody.

The immunological events following initial exposure to EHV-1 can be summarised as follows: 1) At the mucosal surface humoral and cell mediated responses are likely to occur. The mucosal humoural immune response has not been fully characterised although it is likely that neutralising Ig appears in nasal secretions. CD8+ T lymphocytes are released into the bronchoalveolar space 2–3 weeks post infection, although the cytotoxic activity of these cells has not been investigated; 2) In the lamina propria, EHV-1 infects trafficking mononuclear cells which subsequently appear in drainage lymph nodes and the circulation resulting in viraemia. Although the identity of infected mononuclear cells in the lamina propria has not been identified, the viraemic population of cells are predominantly CD8+ T lymphocytes. In the circulation, and presumably in the lamina propria as well, infected lymphocytes express virus antigens only transiently and, from around 3 weeks post infection, virus exists in circulating lymphocytes in an antigenically silent, latent form. Endothelial cells are also infected in the lamina propria and these probably act as another route by which trafficking lymphocytes are infected. The humoral and cellular immune responses in the lamina propria have not, as yet, been fully elucidated although this is one area of current EHV-1 research. Of particular importance, the virus antigens that drive CTL responses in the lamina propria have not been elucidated. This information is likely to provide the key to protective immunity. Related to this, the interaction between dendritic cells and EHV-1 has not yet been studied in detail; 3) In the circulation, high titres of circulating antibody develop, directed mainly against virus glycoproteins gB, gC and gD. There is a short-lived CF antibody response and a more durable neutralising antibody response. However, neutralising antibody titres do not correlate with protection and do not shorten the duration of viraemia, although high titres of circulating antibody may correlate with reduced duration of virus shedding from the nasopharynx. Circulating CD8+ class 1 restricted CTL and CTL precursor (memory) cells also appear in the circulation. CTLp cells persist in the circulation for months after infection and low frequencies of CTL memory cells may be associated with increased susceptibility to infection; 4) In other organs, notably the uterus and CNS, virus is transferred from trafficking lymphocytes to endothelial cells inducing thrombosis, ischaemia, possibly virus translocation into the foetus and, rarely, neurones of the CNS. The immune responses during this phase of infection have, for reasons of practical difficulty, not been studied in detail. It is known that endothelial cells in the uterus express virus antigens, making these candidate targets for clearance by CTL. Whether virus transfer from lymphocytes to endothelial cells occurs by extracellular release of virions or by fusion of infected cells is unknown. Thus, the role of antibody and CTL responses in controlling this phase of infection is not understood. It is possible that CTL responses targeting infected endothelial cells could result in immunopathological damage to the uterus and CNS and this is the basis for the debate about the clinical use of corticosteroids in the management of paresis.

**Latent infection**

The immune control of latency and reactivation is less well understood. It is likely that the lytic and latent infection cycles are separate and the latency is established in the early stage of infection in parallel to the lytic infection cycle. In other words, the outcome of infection of a cell is either lytic infection and death, or latent infection and survival. It is not known what role, if any, the immune response plays in this process.

It is well established that latent infections are established in mononuclear cells, mainly CD8+ T lymphocytes, in both drainage lymph nodes and in the circulation; and that latency in these cells persists for lengthy periods, probably for the life of the horse. Latent infections are also established in trigeminal ganglionic neurones. Latently infected cells do not express virus antigens and are not susceptible to immune surveillance. Thus, systemic virus neutralising antibody (VNA) titres and presumably the frequency of CTL memory cells gradually declines during latency.

The major role of the immune system probably lies in controlling virological events during reactivation, although this area has not received detailed study for EHV-1 infections. It is known that reactivation events are marked by increases in circulating VNA titres, together with shedding of infectious virus from the respiratory tract and
viraemia. Immune responses probably act not by determining whether virus reactivates from lymphocytes or trigeminal ganglionic neurones but by limiting the extent of replication of virus once it has reactivated. Thus, humoral immune responses act at the respiratory epithelial surface mucosa by neutralising free virions via VNA and cellular immune responses eliminate infected cells via CTL responses. In the circulation, reactivating lymphocytes may transiently express virus antigens, rendering them susceptible to clearance by CTL.

The mechanism of transfer of reactivating virus from circulating latently-infected lymphocytes to the respiratory epithelium and to uterine and CNS endothelium is not understood. The opportunity for the immune response to intervene in this process is thus also not understood.

**Areas that require further study**

There is general consensus that circulating antibody responses (CF and VNA) are not the sole determinants of immunity to EHV-1. There is no correlation between either vaccine-induced or natural infection-induced circulating antibody levels and protection from infection or reinfection; and viraemia occurs in the face of neutralising antibody. CTL response is therefore the area of immune response that urgently requires further study.

**There are 4 key questions**

1. Which virus antigens are responsible for driving CTL responses in the *lamina propria* and in the circulation (including the interactions between EHV-1 and dendritic cells)?

2. What is the role of CTL in the termination and prevention of EHV-1 induced viraemia?

3. Can the measurement of CTL provide a quantitative measure of protection or susceptibility to infection?

4. Do CTL responses provide an immunological means of preventing transfer of virus from lymphocytes to endothelial and epithelial cells?

**Two further important immunological aspects are worthy of study**

1. Mucosal antibody responses to infection and correlation between these and protection.

2. The role of mucosal and systemic antibody and CTL responses in controlling latency and reactivation.

Two key techniques have been developed that provide an opportunity for more accurate dissection of the cellular immune responses to EHV-1: 1) estimation of CTLp frequency by limiting dilution analysis; and 2) semi-quantitative and quantitative measurement of equine cytokine mRNA. However, although these techniques are being applied both to peripheral blood samples and to tissues collected post mortem, it remains to be seen whether endoscopic biopsies will provide adequate tissue samples for the study of *lamina propria* and other tissue CTL responses in the live horse.

**Acknowledgements**

The ideas presented in this presentation are the result of collaborative projects with Drs. Julia Kydd and Ken Smith of the Animal Health Trust, Newmarket, UK and Dr George Allen of the University of Kentucky, USA. Grant support for the work discussed here was provided by the Equine Virology Research Foundation, the Horserace Betting Levy Board, The Wellcome Trust, The BBSRC and the Grayson Jockey Club Research Foundation.
It is generally accepted that horses acquire an immunity to nematode parasite infections. Some of these responses such as those to Strongyloides westeri and Parascaris equorum occur rapidly in foals, are complete and long lasting. Most others are not. Detailed studies on acquired resistance to nematode infections of horses is, however, limited and pertains almost exclusively to strongyle infections. Based on their biology it is not surprising that the acquisition and character of immunity to infections of the large strongyles, specifically Strongylus vulgaris and the small strongyles or cyathostomes differ markedly. What is known about both will be summarised briefly (Klei 1992, 2000).

Although the large strongyles of equids are the most pathogenic nematodes found in the horse they are controlled readily by the use of the macrocyclic lactone anthelmintics, ivermectin and moxidectin. On closed, well managed, horse farms these parasites are no longer considered to pose a significant health problem. Nonetheless, infections of parasite free ponies with known numbers of S. vulgaris infective larvae remains a useful model to study equine immune responses to nematode parasites and the most detailed information on equine immunity to nematodes has come from this model system (Klei 1992, 2000). The concomitant immunity to S. vulgaris where both adult and larval stages of the parasite live unaffected within the lumen of the caecum and mesenteric vasculature while incoming infective third stage larvae (L3) are killed, is acquired early after initial exposure to L3. In this system a strong acquired immunity can be induced by vaccination with 2 doses of orally administered irradiated L3. This vaccination provides >90% protection against challenge infection, prevents arterial lesions and protects completely from clinical signs associated with the early arterial migrations of these parasites. This protection has been associated in secondary infections with an anamnestic eosinophilia which does not occur in primary infections until the parasites are established. In vitro culture of L3 with immune serum, or Ig from immune serum, mediates the adherence of cells to the surface of the L3. This adherence immobilises and kills the larvae if the cell mixtures contain eosinophils from eosinophilic horses. Neutrophils or macrophages do not mediate this reaction. Eosinphils from eosinophilic ponies infected with S. vulgaris have been demonstrated to possess increased numbers of Fe and complement receptors and be hypodense, all of which are indicators of an activated state. Only these activated eosinophils, and not normal eosinophils, are active in the in vitro ADCC of L3. The antibodies which mediate this response are species- and stage-specific. Although these data suggest a role for antibody in the protective immunity seen, passive transfer of hyperimmune serum does not protect from challenge infection. However, this passive transfer does reduce the severity of intravascular lesions while promoting a marked and significant perivascular inflammation which is predominately eosinophilic. An anamnestic eosinophilia was also seen in the ponies which received hyperimmune serum but not controls. These observations indicate that a cellular immune response was central to this protective immunity. Early studies demonstrated that lymphocytes from immune ponies cultured with soluble somatic antigen of S. vulgaris produced a factor(s) which was chemotactic for eosinophils in vitro. The stimulation of factor production was species specific in that similar antigens produced from S. edentatus did not induce chemotatic factor induction in immune lymphocyte cultures. Interestingly, field studies using the irradiated L3 vaccine also showed a species specificity when
vaccinated ponies were challenged with mixed species of Strongylus. The character of this T-cell response has been partially defined in this model by measuring the in situ and in vitro expression of cytokine genes using quantitative RT-PCR measurements of cytokine mRNA in peripheral blood mononuclear cells (PBMC) and colonic lymphocytes. These protective immune responses coincide with the induction of a marked increase in gene expression of IL-5 but not IL-4 or IFN-γ in PBMC and colonic lymph nodes (Swiderski et al. 1999). Clear differences have yet to be seen in the compartmentalisation of this response to the large intestine. Earlier studies have shown that vaccination with somatic antigens of S. vulgaris in RIBI adjuvant induced a response to challenge infection which was more severe and characterised by an antibody response in the absence of an anamnestic eosinophilia, and a dominance of IFN-γ mRNA. Attempts have been made to alter the effectiveness of the irradiated L3 vaccination by superimposing the RIBI vaccination, and its associated Th-1 IFN-γ response, upon the irradiated L3 vaccination. Reductions were seen in eosinophilia and IL-5 mRNA by pre-treatment with the RIBI immunisation which induced elevated levels of IFN-γ mRNA. However, these effects were insufficient to alter the protective immune responses to challenge infection. Data from these later experiments are still being collected and analysed. Nonetheless, alternative approaches will be necessary to define the requirements for eosinophils and associated Th2 responses in the protective immunity seen in this system.

Unlike the large strongyles the cyathostomes continue to be found in horses on well managed farms and have developed resistance to all classes of anthelmintic except the macrocyclic lactones. Concern is increasing over the potential development of macrocyclic lactone resistant populations of cyathostomes. Data on field observations suggest that horses acquire immunity to cyathostome infections with age (Klei and Chapman 1999). When examining this resistance using strongyle faecal egg counts, it is clear and not surprising that horses respond to strongyle nematode infections like other hosts. The frequencies of EPGs are evenly dispersed within the yearling population but over dispersed in the mature horse population. In this situation very few mature animals are infected with large numbers of parasites. It is likely this difference in acquired resistance is genetically regulated. Examination of adult worm recoveries from ponies selected because they were infected, i.e. they were examined post mortem as controls in anthelmintic efficacy trials, indicates that although this selected older population had worm numbers similar to those of younger individuals, the EPG were significantly lower than in the young animals. This suggests that some immune response affects the fecundity of the adult worms without affecting its survival. In general, the protective immune response against cyathostomes is slow to develop and incomplete. Evidence from experimental infections is more convincing and indicates that resistance is acquired with exposure and is directed against all stages of the parasite life cycle. Some immunity against developing larval stages in the mucosa, however, appears to require less exposure and is acquired early. Protection against invasion or establishment of the early L3 is only seen in older chronically infected animals. Infection with L3 induces some non-specific events which cause the expulsion of existing lumen dwelling adults. This ‘self cure’ like phenomenon is not species-specific. Antibody responses to somatic extracts of parasites, not surprisingly, do not correlate with protective immunity. Serendipitous observations have suggested that an increase in IL-4 expression is associated with the spontaneous expulsion of adult parasites. However, this has yet to be demonstrated in controlled experiments. Further examination of this experimental model is required to define the immune response to these parasites.

**References**


By far the majority of immunological effort is focused on both innate and adaptive immune responses that occur at sites of infection within the respiratory, gastrointestinal and other mucosal surfaces (McGhee and Kiyono 1993). These mucosal reactions can occur independently of systemic immunity or they may act in concert. Thus, pathogen-specific antibody detected at mucosal surfaces can result from local secretion by recruited antibody secreting cells (ASCs) or transudation of antibody from plasma. Typically, after initial stimulation of lymphocytes at the site of infection (the induction site), lymphocyte activation at local lymph nodes occurs. These activated cells (including ASCs) enter the blood system and return to the mucosal sites where they secrete antibody (the effector site). Because there is linkage of immune responses at different mucosal sites, antibody secretion may be detected at both the site of initial infection and at other mucosal sites. For example, stimulation of the gut immune system may result in production and detection of specific antibody in the trachea (Mestecky et al. 1994).

Pathogen-specific and other antibodies, particularly of the IgA isotype, are found commonly in secretions of the upper respiratory tract and gut of normal healthy and diseased animals. The predominance of these antibodies results from a combination of events including the high rate of IgA isotype switching in ASCs, their selective localisation and proliferation at mucosal effector sites (Husband et al. 1999). Because of the relatively short half-life of B cells, the continuous supply of antibodies at these sites is achieved by their constant replacement. The regulation of this process is not only by mucosal CD4+ lymphocytes in the sub-mucosae but, significantly, by mucosal epithelial cells. These produce many cytokines including those that are critical for maturation of B cells and IgA secretion such as TGF-β and IL-6, as well as IL-2 and IL-10 (Ehrhardt et al. 1992; Salvi and Holgate 1999).

The histological and functional features of the equine mucosal immune system were first described by Mair et al. (1987a) and these showed many similarities with other species. As with other species (Neutra and Kraehenbuhl 1992), the equine microfold (M) cell is a critical component in the cascade from antigen deposition at mucosal surfaces to transepithelial transport and development of mucosal immunity. Antigens are transported into mucosal lymphoid tissues by M cells, which are only present in the follicle-associated epithelium overlying organised lymphoid tissue in the nasal and oral cavities as well as the intestine and bronchi.

Several studies have demonstrated immunoglobulins in the respiratory secretions of normal horses (Mair et al. 1987b) and virus-specific IgA and IgG antibodies in the upper respiratory tract of horses after influenza infection (Hannant et al. 1989). Further support for the function of a mucosal immune system in the horse has come from the demonstration of traffic of virus-specific ASCs in the blood (en route from induction site to effector sites) after virus infection or intranasal vaccination (Hannant et al. 1996). The detection of virus-specific antibody in nasal wash samples 2–3 days after the transient appearance of ASCs in the blood, confirmed there was a locally-recirculating mucosal immune system in the horse (Hannant et al. 1999). Exploitation of this knowledge in horses has occurred by experimental studies using inactivated virus vaccines given intranasally (with cholera toxin B chain, CTB, or other adjuvants) or mucosal application of virus gene products and/or
DNA (Lunn et al. 1999). CTB is an efficient mucosal adjuvant because not only does it stimulate IgA class-switching, but it upregulates TGF-β activity at mucosal surfaces, which is essential for IgA-secreting B cell maturation and expansion (Kim et al. 1998).

This work has opened many possibilities for the rational design of improved vaccines for equine respiratory pathogens. One potential application derives from the observations that in other species, the mucosal immune system of neonates may be primed under cover of maternal antibody. This may offer a route to overcome the well-known interference of systemic immunological priming in foals.

There is great potential now for mucosal vaccination of horses with inactivated antigens or DNA of pathogens in combination with exogenous cytokines. More than one vaccine manufacturer is investigating intranasal presentation of vaccine antigens in horses as a route to boost mucosal immunity. It is to be hoped that this approach will be exploited further to take the best advantage from stimulation of this important immune mechanism.

REFERENCES


MUCOSAL ANTIGEN DELIVERY IN HORSES

J. F. Timoney and A. S. Sheoran

Gluck Equine Research Centre, University of Kentucky, Lexington, Kentucky 40546-0099, USA

Inductive sites in the nasopharynx are an attractive target for new generation vaccines because of their accessibility and absence of chemical barriers such as acidity and hydrolytic enzymes which might degrade immunogens administered per os. However, an intranasal vaccine must be presented in a manner that will circumvent mucociliary clearance in the nasopharynx and resist the mechanical scouring of the lingual and palatine tonsillar surfaces that accompanies swallowing. The vaccine must not be affected by naturally occurring polyreactive IgA, must not induce tolerance and should induce a memory B and T lymphocyte response. Intranasal delivery systems evaluated in the horse include microparticle encapsulation, a mucoadhesive carrier, a cholera toxin chimera and an avirulent salmonella.

MICROPARTICLE ENCAPSULATION

Microencapsulation involves coating of antigen with a biodegradable polymer such as poly DL-lactide-co-glycolide so that, after antigen release in associated lymphoid tissue, mucosal and systemic antibodies are elicited. However, ponies immunised with 250 µg of an immunogenic peptide (SeMF3) of the SeM protein of S. equi on Days 0, 7 and 42 made no detectable serum antibody. SeM specific mucosal IgA responses were detected in 2 vaccinates on Day 21 and in all 3 on Day 49. The absence of a systemic response may have been due to failure of release of antigen from the mucosal compartment.

MUCAADHESIVE COMPOUNDS

Bioadhesive polymers such as sodium alginate have been used for mucosal application of killed influenza vaccine to elicit specific mucosal and systemic antibody responses. Non-polymeric (food grade) sucrose acetate isobutyrate (SAIB) is a highly viscous excipient which becomes much less viscous in appropriate solvents including ethanol in which it can be aerosolised readily. After deposition on the nasopharyngeal mucosa, the solvent evaporates and the SAIB and dissolved antigens form a sticky film which degrades very slowly. Adult Thoroughbred mares were inoculated intranasally with 200 mg SeMF3 in 2 ml 75:25 SAIB-ethanol solution on Day 0 and again 28 days later. Control mares were given 200 mg SeMF3 alone. Of the mares vaccinated with SeMF3-SAIB 91% had made SeM specific serum and mucosal IgA by Day 42. Control mares made only short-lived mucosal and no serum responses. Interestingly, serum and mucosal antibodies of the same mares showed different patterns of reactivity with linear epitopes of SeM consistent with the conclusion that SAIB was effective in delivery of SeMF3 to both the mucosal and systemic immune compartments.

CHOLERA TOXIN-SeMF3 CHIMERAS

Cholera toxin (CT) is a potent mucosal immunogen and adjuvant. The pentameric B (CTB) subunit binds to GM1 gangliosides on epithelial cells and M cells thereby enhancing its own uptake by mucosal associated lymphoid tissue. Ponies inoculated intranasally with a CT-SeMF3 chimera containing the entire ctB gene, the nontoxic A2 region and SeMF3 made strong CTB and SeMF3 specific serum IgGb responses after the first immunisation but no subsequent serum antibody responses. In contrast, specific mucosal IgA responses were boostable but varied in time of onset for each pony and did not attain the amplitude observed for convalescent responses.
The failure to stimulate anamnestic serum antibody responses is unexplained and is a serious limitation of the CT-chimera approach.

**AVIRULENT SALMONELLA TYPHIMURIUM MGN 707**

Avirulent mutants of *S. typhimurium* are attractive mucosal vaccine delivery systems because of their safety and ability to stimulate both the mucosal and systemic immune compartments. MGN 707, a Δ cya Δ crp mutant of an equine isolate of *S. typhimurium*, genetically engineered for temperature regulated expression of SeMF3, was inoculated intranasally into ponies on Days 0, 35, 56 and 161. Strong SeM specific serum responses occurred in all ponies and delayed but strong mucosal IgA responses were noted in 4 of 6 ponies. Induction of mucosal antibody responses required induction of SeMF3 expression prior to administration. Serum and mucosal responses to salmonella antigens and to SeMF3 were boostable suggesting that pre-existing antibody does not cause immune exclusion.
REGULATION OF MUCOSAL IMMUNE RESPONSES

G. Soboll, D. W. Horohov*, C. W. Olsen and D. P. Lunn

School of Veterinary Medicine, University of Wisconsin, Madison, Wisconsin; * Louisiana State University, Baton Rouge, Louisiana, USA

Equine influenza is endemic in Europe and the USA and remains one of the most common infectious diseases in the horse. However, current vaccination programmes are largely ineffective (Mumford 1992; Morley et al. 1999) Nelson et al. (1998) showed that inactivated vaccines offered no protection 3 months after vaccination and induced non-protective IgGT antibody isotype responses. In contrast, natural infection is associated with serum IgGa and IgGb in addition to mucosal IgA, and protects from re-infection for up to one year. Lunn et al. (1999) showed that DNA vaccination with the equine influenza hemagglutinin gene protects horses from homologous challenge infection and induces appropriate IgG isotype responses. However, this approach failed to induce a mucosal IgA response.

An interesting observation made in an initial DNA vaccination experiment (Lunn et al. 1999) was that, in the absence of a mucosal IgA response, the production of mucosal IgGb appears to be critical for protection. Although appropriate IgG responses are often sufficient for protection against influenza, induction of a mucosal IgA response remains important and may improve protection in terms of heterotypic immunity and better control of viral shedding. An attempt was made to elicit mucosal IgA production by co-administration of IL-6 DNA and hemagglutinin DNA (HA DNA). This experiment failed to switch the immune response at the mucosal level but did indeed switch the immune response at the level of serum IgG responses, as indicated by up-regulation of serum IgG(T) and down-regulation of serum IgGb responses (Soboll et al. 1999).

For induction of mucosal IgA production it is critical to stimulate the mucosal associated lymphoid tissue (MALT) directly (Underdown and Mestecky 1994; McGhee and Kiyone 1992). Following a decision to change vaccination strategy, animals were primed with an intranasally administered vaccine. Because intranasal vaccines by themselves do not typically generate IgA (Holmgren et al. 1993, 1994; Tamura et al. 1994), cholera toxin was used as an adjuvant. Cholera toxin has been shown highly efficacious in inducing strong mucosal IgA responses, as well as boosting the immune system in general (Holmgren et al. 1993; Tamura et al. 1990, 1991, 1994; Hirabayashi et al. 1990, 1992; Hornquist and Lycke 1993).

The primary goal of this experiment was to determine the effect of cholera toxin and hemagglutinin DNA co-administration on antibody isotype responses. As a secondary objective, we examined the duration of the immune response to DNA vaccination in terms of protection from challenge infection, antibody responses and the presence of influenza specific memory cells at 3 months post vaccination.

Two intranasal DNA vaccinations were followed by 2 gene gun vaccinations. For the intranasal vaccine the DNA was administered through a narrow gauge catheter positioned in the nasopharynx. The gene gun vaccinations were delivered at skin and mucosal sites using the Powderject XR gene gun. Three groups of 4 influenza negative ponies were established; CT-HA DNA vaccinates, HA DNA vaccinates and controls. For the intranasal vaccines, the CT-HA DNA vaccination group received hemagglutinin DNA mixed with cholera toxin and the HA DNA group received hemagglutinin DNA alone. Both groups were then given 2 further gene gun vaccinations. The control pony group initially comprised 2 sentinel ponies, used to confirm that influenza virus was not accidentally introduced into the pony groups. Fourteen days prior to
challenge infection the number of control ponies was increased to 4. All ponies were challenged by nebulisation of a high dose of homologous virus 3 months after the last vaccination. Antibody isotype responses in serum and nasal secretions were analysed by ELISA. Nasal swabs for virus isolation were collected for 12 days after challenge infection and the egg infectious dose 50%, or EID50, was determined. Immediately prior to challenge infection, PBMCs were prepared from 2 ponies and used to generate dendritic cells. Influenza virus-specific proliferation assays were then conducted using autologous dendritic cells or PBMCs as stimulator cells, which were either infected with influenza virus or transfected with HA DNA using the gene gun.

Following challenge infection, clinical disease was significantly reduced in both vaccination groups when compared to the control group but not significantly different between the vaccination groups. Virus shedding was seen in all groups, nevertheless there was a reduction in the amount and level of virus shed in the vaccination groups, when compared to the control animals. Again no significant differences could be found between the 2 vaccination groups. The IgG isotype responses resembled closely what had been reported in our previous studies. DNA vaccination induced significant IgGa&b antibody titres following vaccination as determined by paired T-tests. However, no significant differences between the 2 vaccination groups in terms of serum IgG responses could be found. In contrast to previous reports a low titred but significant IgA response could be detected in the CT-HA DNA group post vaccination. By the time of challenge infection antibody titres waned, but post challenge infection the CT-HA DNA vaccinates again produced a significantly higher IgA response. An additional experiment was performed in 2 vaccinated ponies prior to challenge infection to measure influenza virus specific memory responses, and to examine differences between dendritic cells and PBMCs as antigen presenting cells. Memory cells were clearly present as indicated by high stimulation indices, but only dendritic cells were effective as stimulator cells when transfected with HA DNA.

Overall, we found that DNA vaccination protects horses from influenza infection but it appears to do so in the absence of a mucosal IgA response. It was implied that mucosal IgGb might play an important role in mucosal immunity in this circumstance. In addition, IL-6 HA DNA co-administration does not switch the immune response at the mucosal surface but it appears to influence the systemic immune response. Lastly, intranasal CT-HA DNA co-administration induces a mucosal IgA response as well as the appropriate IgG responses. This additional IgA response is, however, of low titre and does not result in improved protection of the animal. In conclusion these results represent an improvement over current inactivated vaccines. However, we believe the responses must be amplified further to produce longer lasting protection with practical value in the field.

REFERENCES


isotype-specific antibody responses to equine influenza virus infection versus conventional vaccination. Vaccine 16, 1306-1313.
THE INDUCTION OF EQUINE HERPESVIRUS-SPECIFIC ANTIBODIES IN THE UPPER RESPIRATORY TRACT OF THE HORSE

C. C. Breathnach, M. R. Yeargan, A. S. Sheoran and G. P. Allen

Department of Veterinary Science, Gluck Equine Research Center, University of Kentucky, Lexington, Kentucky 40546-0099, USA

INTRODUCTION

Equine herpesvirus-1 (EHV-1) is a leading cause of viral abortion in mares despite the availability of commercial vaccines. The pathogenesis of EHV abortion is a complex series of events (Allen et al. 1999). The virus initially infects and replicates in the upper respiratory tract epithelium. Progeny virions can then infect lymphocytes and monocytes in the underlying subepithelial tissue and draining lymph nodes, giving rise to a pool of infected leucocytes which can establish viraemia. This leucocyte-associated viraemia is critical in the pathogenesis of EHV-1-induced abortion, as it allows contact between infectious virus and the permissive endothelium of the uterine vasculature, where subsequent vasculitis or transplacental spread of infectious virus to the fetus can lead to premature fetal expulsion.

The mucosal immune system offers the potential for immune exclusion of virus infection at the respiratory tract epithelium portal of entry (Wilkie 1982), thereby precluding the establishment of infection. Little is known about induction of local EHV-specific antibodies and their role in this first line of immunological defence against EHV abortion. This represents a fundamental gap in our understanding of the effector components of EHV-1 immunity and hinders our ability to stimulate more effective herpesvirus abortion defence strategies in the horse by vaccination.

EXPERIMENTAL DESIGN

Twenty mixed-breed weanlings were assigned to 5 equal groups so that they were age-matched. Each group received 2 doses of EHV antigen, 3 weeks apart, and all 20 weanlings were challenged simultaneously. Group 5 was divided into 2 pairs of animals. One pair was allowed to become infected with Army 183 by contact exposure on 2 occasions. The other pair remained untreated until final challenge (Table 1).

Inoculation of weanlings with Army 183 and with Rhinomune (Pfizer Animal Health Inc., Philadelphia, USA) was performed in identical fashion. Both antigens were delivered by direct intranasal instillation of 3 ml of a $2.35 \times 10^7$ PFU/ml cell-free suspension of live virus onto the nasal turbinates. Pneumabort K (Fort Dodge Animal Health, Kansas, USA) was administered im. Rectal temperature and nasal discharge, together with virus isolation from nasopharyngeal swabs and heparinised blood samples, were monitored for up to 14 consecutive days post inoculation.

<table>
<thead>
<tr>
<th>Group</th>
<th>No of weanlings</th>
<th>Primary inoculation</th>
<th>Secondary inoculation</th>
<th>Challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>4</td>
<td>Army 183</td>
<td>Army 183</td>
<td>Army 183</td>
</tr>
<tr>
<td>Group 2</td>
<td>4</td>
<td>Rhinomune</td>
<td>Rhinomune</td>
<td>Army 183</td>
</tr>
<tr>
<td>Group 3</td>
<td>4</td>
<td>Rhinomune</td>
<td>Pneumabort K</td>
<td>Army 183</td>
</tr>
<tr>
<td>Group 4</td>
<td>4</td>
<td>Pneumabort K</td>
<td>Pneumabort K</td>
<td>Army 183</td>
</tr>
<tr>
<td>Group 5a</td>
<td>2</td>
<td>Army 183 (contact)</td>
<td>Army 183 (contact)</td>
<td>Army 183</td>
</tr>
<tr>
<td>Group 5b</td>
<td>2</td>
<td>None</td>
<td>None</td>
<td>Army 183</td>
</tr>
</tbody>
</table>
MEASUREMENT OF NASAL WASH IMMUNOGLOBULINS

Total IgA in each nasal wash sample was measured by ELISA by coating 96-well plates with an affinity purified sheep-anti-equine IgA polyclonal antiserum (Bethyl Laboratories Inc., Texas, USA). Nasal washes were titrated in duplicate columns on each plate, and immunoglobulin reference serum standard (Bethyl Laboratories Inc.) was titrated in the remaining pair of columns, such that IgA was at a starting concentration of 1 mg/ml. The reference serum allowed a standard curve for IgA content to be set up for each plate. Using the standard curve, OD490 readings corresponding to the amount of total IgA in each nasal wash could be translated into milligram quantities of IgA per millilitre of nasal wash. This served as an internal control for normalising virus-specific immunoglobulin isotype levels.

Individual antibody isotype responses to EHV were measured in nasal wash samples using monoclonal antibodies (mAbs) specific for equine IgGa (CVS 45), IgGb (CVS 39), IgG(T) (CVS 38), IgA (BVS 2) (Sheoran et al. 1997, 1998) and IgM (CM7; Custom Monoclonals International, California, USA). Ten columns of 96-well plates were coated with whole EHV-1 virus antigen, and the remaining 2 columns with polyclonal isotype-specific capture antibody specific for the isotype being measured for the establishment of a standard curve as above. Using the standard curve, microgram quantities of virus specific antibody of the isotype in question were calculated for each nasal wash. All results were standardised to 1 mg total IgA.

RESULTS

Exposure of seronegative weanling foals to virulent EHV-1 (Army 183) gave rise to the induction of a local mucosal humoral immune response. This was demonstrated by the detection of a wide range of virus-specific antibody isotypes in the nasal washes (Fig 1). Virus-specific IgA was elicited by EHV-1 inoculation, and proved durable in the local environment of the upper respiratory tract. Appreciable levels of EHV-specific IgA were still detectable in the nasal washes on the final sampling day, 13 weeks post final challenge (Fig 1). Virus-specific IgGa and IgGb isotypes were found at high concentrations in nasal washes for brief periods during the acute phase of disease. IgG(T) was generally present in tandem with IgGa and IgGb, but was usually found at lower quantities. This pattern of appearance of IgG species in the upper respiratory tract secretions is suggestive of inflammation-dependent exudation of serum antibody. It seems plausible that the rapid decline in nasal wash IgG species was due to both the restoration of the natural epithelial barrier during convalescence from EHV-1 infection, thereby preventing further passage of IgG into the respiratory lumen, and the short half-life of monomeric immunoglobulins in the nasal cavity.

All 6 seronegative weanlings inoculated with Army 183 (Groups 1 & 5a) showed clinical signs of disease consistent with primary exposure to virulent EHV-1 antigen, following primary experimental inoculation. However, they were protected from upper respiratory tract disease following both subsequent challenges. EHV-specific IgA and other
antibody isotypes were present in the respiratory secretions, albeit at low levels. It seems likely that local antibody against the challenge virus, whether pre-existing or rapidly recalled, contributed to the protective immune response.

Intranasal administration of Rhinomune (Group 2) was not effective in the stimulation of local EHV-specific antibody detectable by ELISA (data not shown). The vaccine strain may have been over-attenuated for this experimental purpose. EHV-specific antibodies of all isotypes remained at baseline levels following both primary and secondary administration of Rhinomune, and remained as such until challenge of the animals with Army 183. A similar situation arose among the animals in Group 3 (Rhinomune intranasally followed by Pneumabort K im) and Group 4 (Pneumabort K im twice; data not shown). Each group of vaccinates demonstrated a reduction in clinical signs following challenge, suggesting that vaccination had conferred some limited protection from clinical disease on those animals, despite its failure to induce detectable levels of pre-challenge local EHV-specific antibody. The post challenge local antibody response of the individuals in these groups was clearly dominated by IgA. The amount of virus-specific IgG isotypes in the nasal washes remained low, presumably because reduced clinical signs resulted in less epithelial damage.

Pathogen-specific antibody present at various mucosal surfaces is reported to protect from several viruses (Zeitlin et al. 1999) including herpesviruses (Israel et al. 1992; Whaley et al. 1994). The demonstration of induction of nasal IgA in the horse following experimental inoculation with EHV-1 suggests a role for this antibody as a first line of defence against colonisation by this important upper respiratory tract pathogen. Previous studies suggested that IgA is the most important antibody isotype at the nasal mucosa for protection of horses from other notorious upper respiratory tract pathogens (Sheoran et al. 1997; Nelson et al. 1998; Hannant et al. 1999).

REFERENCES


The term 'immunogenetics' has enjoyed many meanings over the past century, ranging from the practical use of antibodies to detect polymorphic gene products to the early, arcane studies of the genes controlling tissue transplantation. For the purposes of this discussion a broader definition will be employed.

Immunogenetics is the study of genes affecting and controlling immune responses. Today this term encompasses complementary investigations of the structure, function, and polymorphism of these genes. Some of the genetic regions influencing immune responses are well-known, although not yet completely understood. These include the major histocompatibility complex (MHC), and the immunoglobulin and T cell receptor regions. Others, such as the genes encoding the cytokines and their receptors, are less well-studied. Progress towards sequencing the entire human genome and the development of comparative gene maps of many species, including the horse, allows construction of a genomic map of the equine immune system. The rapidly expanding array of molecular tools developed by the Horse Genome Project will enable sophisticated studies of the function of genes of the equine immune system and applications in production and disease.
Immunoglobulin (Ig) effector functions during immune response are dependent on the isotypes which are expressed after B-cells have recognised their specific antigens. Whether immunoglobulins induce protection, have no effect on the outcome of the immune response or mediate autoaggression or allergy is influenced by the isotype.

Many studies have been performed to characterise equine Ig isotypes using serological and biochemical methods (Table 1). These studies allowed the description of IgM, 5 to 6 IgG isotypes, IgE and one or 2 IgA subclasses, but particularly for IgG and IgA the exact number of isotypes could not be determined. To resolve the dilemma of how many isotypes exist in the horse, the molecular basis of equine Ig isotype expression was investigated.

Ig isotypes are encoded by the constant heavy chain genes (CH-genes), which are located on the immunoglobulin heavy chain gene locus (IgH-locus). The IgH-locus contains the 5’ located variable, diversity and joining genes (VDJ genes), representing the gene segments encoding the variable domain and the different CH-genes (Fig. 1). The IgH-loci of mouse and rat are very similar having 5’ located Cµ and Cδ genes, 4 Cγ genes clustered together and one Cε and one Cα gene at the 3’ ends (Shimizu et al. 1982). In contrast, the human IgH-locus shows a duplication of a γ−γ−ε−α fragment which occurred during evolution. This leads to the expression of four Cγ genes, one Cε gene, but 2 Cα genes in man (Planagan and Rabbitts 1982). Further, the rabbit constant heavy chain genes are completely different, with only one Cγ gene, but 13 Cα genes.

To analyse the equine constant heavy chain genes we used human and murine probes to isolate equine Cγ, Cε and Cα genes from a genomic phage library. One Cε gene and one Cα gene were isolated and clustered together on one DNA fragment, represented by 4 overlapping phage clones (Wagner et al. 1997). In addition, 6 different Cγ gene containing clones were obtained by the same rationale. Subsequently, these equine CH-genes were used as DNA probes to analyse equine genomic DNA from PBMC by restriction analysis. The restriction analysis confirmed the existence of 6 equine Cγ genes, one Cε and one Cα gene.

To align the 6 Cγ genes, which did not overlap, and to determine their relative position to the Cε-Cα cluster, as well as to the equine Cµ gene, deletion analysis of heterohybridomas expressing equine IgM and IgG was performed (Wagner et al. 1995).

The deletion analysis was based on class switch recombination, which occurs during B-cell development. The isotype expression is known to be regulated by different cytokines which are produced by T-helper cells (Fig 2). Cytokines secreted by T-helper 1 cells, like IFN γ induce the expression of isotypes other than

**TABLE 1: Equine isotypes characterised by serology or biochemistry**

<table>
<thead>
<tr>
<th>Ig</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM</td>
<td>Hill and Cebra (1965); Montgomery (1973)</td>
</tr>
<tr>
<td>IgGa, IgGb, IgGc</td>
<td>Rockey (1967); Montgomery (1973)</td>
</tr>
<tr>
<td>IgG(T), with probably 2 subclasses</td>
<td>Weir and Porter (1966); Helms and Allen (1970); Sheoran and Holmes (1996)</td>
</tr>
<tr>
<td>AI/IgG(B)</td>
<td>Helms and Allen (1970); Seide et al. (1987)</td>
</tr>
<tr>
<td>IgA</td>
<td>Vaerman et al. (1971); Pahud and Mach (1972)</td>
</tr>
<tr>
<td>IgE</td>
<td>Suter and Fey (1981); Halliwell and Hines (1985)</td>
</tr>
</tbody>
</table>
T-helper 2 cytokines, like IL4. For example, IL4 induces expression of murine IgG1 and in higher concentrations to IgE. In contrast, IFN-γ inhibits both steps, but induces IgG3 and IgG2a expression (Siebenkotten and Radbruch 1995).

Early in the B-cell development, the variable genes on the IgH-locus on one chromosome undergo a VDJ recombination to express a functional variable heavy chain domain together with the first 3’ located constant gene, the Cµ gene. This leads to the expression of IgM on the cell surface, as well as to IgM secretion.

The class switch recombination is initiated when the B-cell recognises a specific antigen. The class switch occurs between highly repetitive intron sequences, known as switch regions, which are located at 5’ of the associated CH-gene. A part of the Sµ region and the switch region of the newly expressed CH-gene are re-arranged and the DNA between both switch regions is looped out and becomes deleted from the chromosome.

In heterohybridomas expressing a defined isotype, only the expressed gene and the 3’ located constant genes are present while all genes at 5’ of the expressed one are deleted. Deletion analysis of equi-murine heterohybridoma DNA, using the equine DNA probes for Cγ, Cε and Cα genes lead to the alignment of the equine CH-genes (Fig 3), with the 5’ located Cµ gene, 6 Cγ genes clustered together, one Cε and one Cα gene at the 3’ end. The designation of the equine Cγ genes as Cγ1 to Cγ6 was carried out according to their position from the 5’ to 3’ direction. The corresponding isotypes for 3 of the 6 Cγ genes were determined according to heterohybridoma expression: Cγ1 encoding IgGa, Cγ3 IgG(T) and Cγ4 IgGb (Wagner et al. 1998).

In addition, the serological data strongly indicate that the remaining Cγ genes are also expressed.

The genetic data offer general information that can be used for equine Ig isotype diagnosis in several diseases. In addition, the analysis of functional aspects like cytokine regulation of isotype expression or the characterisation of the equine constant heavy chain gene evolution by nucleotide or amino acid sequence comparison can be performed.

We decided to use the DNA for the expression of rare equine isotypes, such as recombinant proteins like IgE. The equine IgE was expressed as a complete chimeric Ig molecule in mammalian cells (Fig. 4).

The chimeric IgE contained murine light chains and a murine VH domain, forming 4- (hydroxy-3-nitro-phenyl) acetyl (NP) specific.
antigen binding sites, and a complete equine IgE constant region (NP-IgE). This NP-IgE had a molecular weight of 230,000 daltons, was highly glycosilated and able to induce an immediate skin reaction after crosslinking with antigen. Thus, the recombinant equine IgE is very similar to the native protein and will be a useful reagent for the production of monoclonal antibodies specific for equine IgE.

REFERENCES


MHC class I and class II genes encode membrane-bound molecules whose function is to bind and present peptides to T cells and thus play a key role in the initiation of an immune response and influence susceptibility to diseases. Some MHC genes and gene products exhibit a remarkably high level of polymorphism. The major focus of MHC research in the horse has been identification of homology between the equine MHC and the MHC of other species, characterisation of the MHC genes of the horse and investigation of associations between MHC alleles and disease susceptibility or antibody production.

The equine leucocyte antigen (ELA) class I alleles are still determined serologically and over 20 different alleles have been described at the so-called ELA-A locus. MHC class II typing is now also performed with molecular techniques such as SSCP and PCR-RFLP (reviewed by Bailey et al. 2000). Disease association studies, however, were performed before such information was available, and were carried out with a limited number (5) of serologically determined MHC class II alleles.

The strongest association which has been found so far is the association between the equine MHC class II allele DW13 and susceptibility to sarcoid tumours (Lazary et al. 1994), a skin tumour of the horse caused by a virus closely related or identical to bovine papilloma virus (Ottén et al. 1993). This association has been described in the USA, Sweden and Switzerland. Most interestingly, associations between MHC class II alleles and susceptibility to papilloma virus induced tumours have also been demonstrated in other species such as squamous cell carcinoma in women (Apple et al. 1994) and tumours induced by Shope papilloma virus in rabbits (Han et al. 1992).

An association between ELA and susceptibility to sweet itch, an allergic dermatitis response to bites of midges and/or black flies, has been described in Icelandic horses (DW22; Halldórsdóttir et al. 1991) and in some Swiss Warmblood horse families (DW23; Lazary et al. 1994).

Two studies suggest that the antibody production against specific antigens is influenced by the MHC in the horse: in the first study, weak associations (0.1>P>0.01) between certain ELA alleles and antibody titres against influenza 1 and 2, equine herpesvirus-1 and ovalbumin were found (Bodo et al. 1994). In the second study (Eder et al. 2001) a possible association between MHC class I antigens and mould-specific IgE antibody levels was investigated. Significant associations (P<0.01) were found between ELA-A8 and low IgE titres against some recombinant *Aspergillus fumigatus* and *Alternaria alternata* allergens. Furthermore, ELA-A1 was associated with higher titres and ELA-A14 with lower IgE titres against mould extracts (P<0.05).

**Acknowledgements**

This work was supported by the Swiss National Science Foundation grant No. 31-49618.96 and by the Hans-Sigrist Foundation of the University of Berne.

**References**


E. Marti

Division of Immunogenetics, Institute of Animal Breeding, Bremgartenstrasse 109A, 3012-Berne, Switzerland

Immunology in 2001
A SYNDROME OF ANAEMIA, IMMUNODEFICIENCY AND PERIPHERAL GANGLIONOPATHY IN FELL PONY FOALS

M. A. Holmes, S. F. E. Scholes*, A. Holliman† and P. D. F. May**

University of Cambridge; *Veterinary Laboratory Agency, Lasswade, Edinburgh; †Veterinary Investigation Centre, Penrith; **Rowcliffe House Veterinary Hospital, Penrith, UK

INTRODUCTION

Fell ponies were used extensively in Cumbria as draft animals but their numbers decreased markedly after the Second World War, although they remained an integral part of fell farming. In recent years there has been a marked increase in the number of breeding mares on fell farms because the breed has become popular for riding and showing. Recently an unexplained progressive syndrome of Fell pony foals has been seen. Despite intensive supportive therapy, foals die usually between 2 and 3 months of age (Scholes et al. 1998). At 2 to 3 weeks of age affected foals are bright and alert but many have diarrhoea, cough and fail to suck. The latter is associated with frequent chewing movements, halitosis and a pale pseudomembranous lingual coating. The diarrhoea and coughing initially respond to treatment but then become unresponsive and persist. The foals become clinically anaemic with dry staring coats, usually at 4 to 8 weeks of age. They deteriorate and most die or are destroyed, between one and 3 months of age.

TABLE 1: Summary of clinical pathology in 3 affected Fell ponies (1, 2, 3), a foal with an unrelated neuropathy (N) and 2 control animals (A, B)

<table>
<thead>
<tr>
<th></th>
<th>Affected foals</th>
<th>Foal N</th>
<th>Healthy foals</th>
<th>Reference range*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>A</td>
</tr>
<tr>
<td>Age (weeks)</td>
<td>24</td>
<td>42</td>
<td>49</td>
<td>44</td>
</tr>
<tr>
<td>Sex</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>M</td>
</tr>
<tr>
<td>WBC (10^12/litre)</td>
<td>2.97</td>
<td>6.39</td>
<td>1.95</td>
<td>3.46</td>
</tr>
<tr>
<td>PCV (litres/litre)</td>
<td>0.16</td>
<td>0.27</td>
<td>0.09</td>
<td>0.16</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>5.8</td>
<td>9.9</td>
<td>4.3</td>
<td>5.9</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>554.5</td>
<td>42.7</td>
<td>46.2</td>
<td>45.0</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>19.5</td>
<td>15.4</td>
<td>22.1</td>
<td>17.0</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>35.8</td>
<td>36.2</td>
<td>37.8</td>
<td>37.8</td>
</tr>
<tr>
<td>WBC (10^9/litre)</td>
<td>6.7</td>
<td>12.3</td>
<td>41.1</td>
<td>29.2</td>
</tr>
<tr>
<td>Lymphocytes (10^9/litre)</td>
<td>1.07</td>
<td>1.47</td>
<td>3.28</td>
<td>1.75</td>
</tr>
<tr>
<td>Neutrophils (10^9/litre)</td>
<td>5.36</td>
<td>9.1</td>
<td>36.9</td>
<td>23.9</td>
</tr>
<tr>
<td>Monocytes (10^9/litre)</td>
<td>0.27</td>
<td>1.72</td>
<td>0.82</td>
<td>3.5</td>
</tr>
<tr>
<td>Eosinophils (10^9/litre)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total protein (g/litre)</td>
<td>48</td>
<td>60</td>
<td>so</td>
<td>65</td>
</tr>
<tr>
<td>Albumin (g/litre)</td>
<td>23.6</td>
<td>29.7</td>
<td>20.9</td>
<td>29.4</td>
</tr>
<tr>
<td>Globulin (g/litre)</td>
<td>24.4</td>
<td>30.3</td>
<td>29.1</td>
<td>35.6</td>
</tr>
<tr>
<td>Urea (mmol/litre)</td>
<td>6.1</td>
<td>49.4</td>
<td>114.0</td>
<td>44.3</td>
</tr>
<tr>
<td>Creatinine (mmol/litre)</td>
<td>114</td>
<td>414</td>
<td>810</td>
<td>796</td>
</tr>
<tr>
<td>Total bilirubin (mmol/litre)</td>
<td>25.4</td>
<td>24.2</td>
<td>37.8</td>
<td>25.2</td>
</tr>
<tr>
<td>Direct bilirubin (mmol/litre)</td>
<td>14.2</td>
<td>8.7</td>
<td>8.5</td>
<td>3.1</td>
</tr>
</tbody>
</table>

*Thoroughbred foals; RBC red blood cells; PCV packed cell volume; Hb Haemoglobin; MCV mean corpuscular volume; MCH mean corpuscular haemoglobin; MCHC mean corpuscular haemoglobin concentration; WBC white blood cells; ND not done.
Immunology in 2001

Haematological and serum biochemical findings in 3 Fell pony foals with the syndrome of anaemia, immunodeficiency and peripheral ganglionopathy, and in 2 healthy control foals and a foal with an unrelated neuropathy are shown in Table 1.

**Clinical Pathology**

Haematological and serum biochemical findings in 3 Fell pony foals with the syndrome of anaemia, immunodeficiency and peripheral ganglionopathy, and in 2 healthy control foals and a foal with an unrelated neuropathy are shown in Table 1.

**Pathology**

**Bone marrow**

The bone marrow of the affected foals was more densely populated with haemopoietic cells and contained fewer lipocytes than marrow from similar sites in the control foals. Late normoblasts were rare in the femoral bone marrow compared to an age-matched control bone marrow. Numerous coarse haemosiderin granules were present in macrophages in affected foals.

**Lymph nodes**

There were sparse to moderate numbers of lymphocytes in the cortices and paracortices of the lymph nodes, and germinal centres were absent from all the lymph nodes examined from these foals. There were macrophages in the sinuses of all the lymph nodes examined from affected foals and they were particularly numerous, together with variable numbers of neutrophils, in inflamed nodes.

**Thymus**

The thymic lobules were very small, there was no clear demarcation between the cortex and medulla, the epithelial structure was prominent and the outer areas of the lobules contained variable numbers of vacuolated macrophages and few lymphocytes.

**Spleen**

Foals had small numbers of periarteriolar lymphocytes and the red pulp was markedly contracted and contained numerous siderrophages; neither germinal centres nor their stromal support were detected.

**Intestine**

No plasma cells were recognised in the small or large intestinal lamina propria of foals, in which the tissue preservation allowed a critical evaluation, and there were no germinal centres in
the gut-associated lymphoid tissue (GALT). There were large numbers of Cryptosporidia on the apical surfaces of enterocytes and, to a smaller extent, of enteroblasts in the duodenum and ileum of some foals. In other foals, the nuclei of some duodenal enterocytes contained large basophilic intranuclear inclusion bodies typical of an adenovirus infection. In contrast, in the control foals, there were many mature plasma cells in the deeper lamina propria of the small and large intestine, particularly in the duodenum and there were prominent germinal centres in the GALT.

Nervous system

All the peripheral ganglia examined contained sparse to moderate numbers of chromatolytic neurones and occasional axonal spheroids. Chromatolysis was usually central but occasionally complete, the latter being accompanied by nuclear pyknosis. Chromatolysis was not detected in the peripheral ganglia of the control foal. No abnormality was detected in the central nervous system.

IMMUNOGLOBULIN ANALYSIS

Isotype concentrations were established using single radial immunodiffusion and the subisotype concentrations were measured using a competition ELISA (Sheoran et al. 1997). The results of the serum immunoglobulin assays are shown in Table 2. The total immunoglobulin levels in the affected Fell pony foals were similar to the control foals. However, the affected foals had relatively higher levels of IgM. The relative concentrations of IgG subisotypes in the serum of the affected foals was also similar to that in the control animals although the absolute levels were, on average, lower.

MEASUREMENT OF PURINE ENZYME ACTIVITIES

A proportion of human SCID conditions result from defects in enzymes involved in purine metabolism such as adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) (Markert 1991; Webster 1987). Conventional biochemical techniques were used to assess ADA, PNP, SAHH and P5N enzyme levels (Peters et al. 1985; Magnusson and Perryman 1980; Morris and Simmonds 1985; Rocchigiani et al. 1992).

Evaluation of purine and pyrimidine pathways was examined by the incorporation of 14C radiolabelled substrates. Substrates were incubated for 14 minutes with cell lysates. The fate of radiolabelled purines, deoxyadenosine and hypoxanthine, and the pyrimidine, orotic acid was established by running product through an anion exchange column using HPLC together with an inline radiation detector. The incorporation or absence of 14C in various pathway products indicated whether or not the major purine metabolic pathways were intact (Table 3).

Purine enzyme activities are generally very low in horses. The PNP levels are higher, if anything, in affected foals. There were low SAHH activities in affected foals. ADA levels were extremely low in all equine samples which provokes a question as to why horses have such low ADA levels.

CONCLUSION

These results suggest that this disease is not a homologue of any well characterised immunodeficiency in any other species. There is no evidence that the syndrome is the same as a SID condition described in Arabian horses (Shin et al. 1997). Although immunoglobulin was present in the circulation of older foals, there is no evidence that this was not derived from the dam via colostrum. There is a clear need for basic genetic and epidemiological studies to establish the heritability and other basic parameters of the disease. Further studies will concentrate on characterising the functional immunodeficiency, bone marrow deficit, and the purine and pyrimidine enzymology.
SESSION II:

New technologies in equine immunity

Chairman: Doug Antczak
Progress in producing monoclonal antibodies (mAbs) for equine immunological study could be well described by a bell-shaped curve. Early progress was slow, then there was a rush of progress charted by 2 workshops. More recently, it has become increasingly difficult to find the resources and the more exacting techniques to focus on specific but pivotal molecules, like IgE and key cytokines. It is hoped that meetings, such as the current workshop, will provide further stimulus.

Large workshops with many new anti-equine reagents are not likely to recur, with the exception of what might be accomplished with proper access to the human antibody body panel. For example, up to 20% of 116 human antibodies cross-reacted with equine cells in a preliminary study conducted at the most recent human leucocyte differentiation antigen workshop. Specific new reagents have been identified since the most recent equine workshop, and include antibodies recognising FcγRIII and FcγRII, CD38, and B7-2 (CD86). In the author’s laboratory, work has been underway, in collaboration with David Horohov of Louisiana State University, to characterise select equine cytokines further. Biological activity for equine IL-6 and IL-10 has been demonstrated, and γ-IFN is currently being investigated. In addition mAbs recognising equine IL-2 have been produced, and γ-IFN is currently being targeted. These efforts have progressed slowly, and further advances will require increased collaborations so that investigators can combine their efforts. To this end a webpage is maintained which can be found at: http://www.vetmed.wisc.edu/research/eirh/. This lists many equine reagents that became available after the last equine workshop, and it will be updated with new information arising from the present meeting.
Rhodococcus equi is an important pulmonary pathogen of young horses. Previous work in a mouse model has shown that blocking IFN-γ in vivo prevents immune clearance of R. equi from the lung. Likewise, adoptive transfer of R. equi-specific CD4+ Th1 cells that produce IFN-γ is sufficient for clearance in immunodeficient nude mice that normally cannot control pulmonary infection. There is also evidence in mice that CD8+ T lymphocytes play a role in protective immune responses. At least part of the effect of CD8+ lymphocytes may be mediated by IFN-γ secretion as well.

The purpose of this study was to develop and validate a flow cytometric method for measuring intracellular IFN-γ in lymphocytes from the horse, and to apply the technique to the study of equine rhodococcal pneumonia. Monoclonal antibodies (Mab) were raised against a synthetic peptide corresponding to the predicted amino terminus of equine IFN-γ (EqIFN-γ) and shown by immunoblotting to react with a full length recombinant EqIFN-γ molecule. One of these antibodies was further shown to detect intracellular IFN-γ in individual lymphocytes following ex vivo stimulation with ionomycin and phorbol 12-myristate 13-acetate (PMA). Briefly, monensin was added to stimulated cultures to block intracellular transport and cause cytokine accumulation in the Golgi apparatus. Upon harvest, stimulated and unstimulated cells were fixed with paraformaldehyde, permeabilised with saponin, and stained with anti-IFN-γ Mab followed by a fluorochrome conjugated secondary antibody. Importantly, the specificity of the IFN-γ staining was demonstrated using synthetic peptide to competitively inhibit binding of the anti-IFN-γ Mab. Staining with Mab to CD2, CD8 and/or CD4 prior to fixation allowed for identification and enumeration of the types of cell producing IFN-γ.

Following work to optimise the assay, we first characterised IFN-γ production by peripheral blood mononuclear cells (PBMC) from normal horses (n=5). As described in other species, no

![Image of flow cytometry results]

**Fig 1:** Example of detecting IFN-γ producing equine T lymphocytes (PBMC) by flow. Percentages indicated in the right quadrants of the second panel are percent of the total cells counted. Percentages described in the text are percent IFN-γ-positive cells for each indicated cell surface marker (eg %CD8+ cells that are IFN-γ-positive).
significant production of IFN-γ was detected in unstimulated cells. However, after stimulation with ionomycin/PMA, the percentage of CD2+ T lymphocytes producing IFN-γ ranged from 13.5% to 45.7% (Fig. 1). This variation is similar to that described in PBMC from man and other species. The percentage of CD8+ lymphocytes producing IFN-γ was 17.6–31.5%, whereas 18.1–47.2% of CD4+ lymphocytes were positive for IFN-γ. As noted in other species, stimulation with ionomycin/PMA resulted in down regulation of surface CD4 expression. Therefore, in subsequent experiments (on cells from the lung) we used tricolour fluorescence and defined CD2+/CD8- cells as putative CD4+ T lymphocytes.

Because all foals are normally exposed to \( R.\ equi \) in their environment, and only a subset develop pneumonia, we have hypothesised that most foals develop protective immune responses. Further, these antigen-specific responses are hypothesised to operate throughout life to prevent rhodococcal pneumonia in adults. In support of this model, work in our laboratory has shown that adult horses efficiently clear \( R.\ equi \) bacteria from the lung in association with an influx of both CD4+ and CD8+ T lymphocytes. The timing and antigen-specific lymphoproliferation of these cells are compatible with a local recall response. A better understanding of the mechanisms of immune clearance in adult horses would help define the requirements for an effective vaccine in foals. Using tricolour fluorescence we determined the phenotype of cells in bronchoalveolar fluid (BALF) producing IFN-γ both before and after challenge with virulent \( R.\ equi \). Only a small number of horses have been tested thus far. Prior to challenge, approximately 10% of CD4+ (CD2+/CD8-) T lymphocytes and 10% of CD2+/CD8+ T lymphocytes in BALF produce IFN-γ when stimulated with ionomycin/PMA ex vivo. Following challenge, approximately 30% of CD4+ T lymphocytes and 30% of CD2+/CD8+ T lymphocytes in BALF produce IFN-γ upon stimulation. Moreover, clearance of a pulmonary challenge is associated with a dramatic increase in the absolute numbers of CD4+ T lymphocytes in BALF that produce IFN-γ upon stimulation (examined at Days 7 and 14 post challenge). In contrast, only small increases in the absolute numbers of CD2+/CD8+ T lymphocytes that produce IFN-γ are observed. Future work will characterise antigen-specific intracellular IFN-γ production associated with clearance of virulent \( R.\ equi \). Our long term goal is to use this information to design a vaccine for use in foals.
IL-4 INDUCED CD23 (FCERII) UPREGULATION IN EQUINE PERIPHERAL BLOOD MONONUCLEAR CELLS AND ALVEOLAR MACROPHAGES

J. L. Watson, K. A. Jackson and J. L. Stott

Departments of Medicine and Epidemiology and Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, California 95616, USA

SUMMARY

The objective of this study was to quantify the induction of equine CD23 transcripts in equine peripheral blood mononuclear cells (PBMCs) and alveolar macrophages cultured with recombinant equine IL-4 (rEQ IL-4). Whole blood was collected from 4 healthy 3-year-old horses and PBMCs were purified over Histopaque. Bronchoalveolar lavage fluid was collected from one healthy horse and alveolar macrophages were purified using adherence to plastic for 120 min. PBMCs and alveolar macrophages were cultured using 4 media conditions: rEQ IL-4 and LPS, rEQ IL-4 only, LPS only and no addition. Total RNA was isolated from cells cultured for 24 and 48 h. Reverse transcribed mRNA was amplified and quantified in real-time polymerase chain reaction (PCR) using a fluorescein labelled internal TaqMan probe. Relative quantification of the CD23 signal was done using the signal for GAPDH, a housekeeping gene, for each sample. Raw data were analysed using the \( \Delta \Delta C_T \) method where the value for each sample was normalised using the corresponding GAPDH value and all values for each condition were calibrated using the untreated control. Without exception, the relative value for CD23 transcripts from PBMCs cultured with rEQ IL-4 and LPS were significantly higher than the untreated controls (P< 0.05). The relative value for CD23 transcripts from alveolar macrophages cultured with rEQ IL-4 was increased more than 1,000-fold when compared to LPS only or untreated controls. In conclusion, rEQ IL-4 induced equine CD23 mRNA transcript upregulation in PBMCs and alveolar macrophages cultured for 24 and 48 h. These findings support a role for equine CD23 in allergic, anti-parasitic and other IL-4 mediated immune responses in horses.

INTRODUCTION

The low-affinity receptor for IgE, CD23 (FceRII), is expressed on lymphocytes, monocytes, eosinophils and platelets. CD23 plays a role in the differentiation and activation of B lymphocytes and is upregulated, or induced, by interleukin 4 (IL-4). There is evidence that the expression of CD23 plays an important role in IgE-associated immune responses, including allergies. Alveolar macrophages from people with allergic asthma express CD23 at a high frequency when compared to non-asthmatics. Horses suffer from a number of allergic conditions in which CD23 may play a role. This study provides evidence that 1) CD23 is present on circulating PBMCs, 2) CD23 can be upregulated on PBMCs by rEQ IL-4 and 3) CD23 can be induced on equine alveolar macrophages by rEQ IL-4.

MATERIALS AND METHODS

Cell isolation and culture

Peripheral blood mononuclear leucocytes (PBML) were harvested from whole blood collected in ACD by centrifugation over Histopaque. PBMLs were washed twice in sterile phosphate buffered saline, resuspended in RPMI at \( 6 \times 10^5/\text{ml} \) and transferred to tissue culture flasks (75 cm\(^2\)). Four culture conditions were used: media alone, supplementation with lps at 10 µg/ml, supplementation with recombinant equine IL4 rich cell supernatant at 12.5%, or both. Cells were cultured at 37°C and 5% CO\(_2\) for 24 or 48 h. Harvested cell pellets were washed twice in PBS and frozen immediately at -80°C.

Cells were harvested from BAL fluid by centrifugation for 20 min at 400 g and washed
twice in sterile Hank’s Buffered Salt Solution. BAL cells were resuspended in RPMI at 2 x 10^5/ml and transferred to tissue culture flasks (25 cm²). Cells were incubated at 37°C and 5% CO₂ for 2 h and non-adherent cells were rinsed out with 2 washes of sterile HBSS. Adherent cells were cultured by the same method as for PBML’s.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from each cell pellet using the Qiagen RNAeasy mini kit. All RNA extractions were treated with RNase-free Dnase I for 30 min. First strand synthesis was performed in a 20 µl volume containing 50 units Superscript II (Invitrogen Life Technologies), 1.25mM random hexamers and 1nM dNTPs.

Real-time polymerase chain reaction (RT PCR) assay

Real-Time TaqMan systems for GAPDH and CD23 were run in separate wells. The PCR reactions contained 400 nM of each primer, 80 nM of each probe, commercially available PCR Mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems) and 5 µl diluted cDNA sample (5-fold dilution) in a final volume of 25 ml. The samples were placed in 96-well plates and amplified in an automated fluorometer (ABI 7700 Sequence Detection System, Applied Biosystems). Amplification conditions were 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C. Transcript quantification was done using the ΔΔC₀ method and is reported as the n-fold difference relative to a calibrator cDNA (CD23 transcription in unstimulated cells).

RESULTS

When PBMCs were cultured with rEQ IL-4 and LPS for 24 or 48 h CD23 signal could be detected and the relative value for CD23 transcripts were significantly higher than the untreated controls, P<0.05 (Figs 1 and 2). The relative value for CD23 transcripts from alveolar macrophages cultured with rEQ IL-4 was increased more than 1,000-fold when compared to LPS only or untreated controls (Fig 3).

CONCLUSIONS

In conclusion, rEQ IL-4 induced equine CD23 mRNA transcript upregulation in PBMCs and alveolar macrophages cultured for 24 and 48 h. These findings support a role for equine CD23 in allergic, anti-parasitic and other IL-4 mediated immune responses in horses.
OPSONISATION BY COMPLEMENT C3 AND IgG AS MEASURED BY A FLOW-CYTOMETRIC IMMUNOASSAY

G. Gröndahl

Department of Large Animal Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden

INTRODUCTION

For a successful phagocytosis by neutrophils, the microbes must be coated with serum opsonins (eg antibodies and complement factor C3b), for which the phagocytes have surface receptors. If the opsonic capacity is low, the risk for infection increases, such as in failure of passive transfer of immunoglobulins in foals.

Knowledge of the mechanisms of complement activation and deposition is important in studies of phagocytosis as well as of immune evading mechanisms of microbes. It is also critical in immune-mediated and inflammatory diseases, and in adverse reactions to artificial surfaces in implants in blood vessels, other soft tissues and hard tissues, eg catheters and bone plates.

When complement opsonisation is of interest, a functional coating assay is more valuable than measuring serum concentrations of the native proteins, or the haemolytic capacity of serum complement. However, traditional haemolytic assays reflect the final result of the full complement cascade reaction and involve many other steps and proteins following initial activation and deposition.

Binding of C3 depends on the conversion of serum C3 to opsonic C3b (Fig 1) by convertases produced by 2 major complement pathways. In the classical pathway, antibodies bound to the antigen initiate the formation of the classical C3 convertase, C4b2a. The alternative pathway, on the other hand, is promoted without the presence of antibodies by spontaneously formed C3b which persists on activating surfaces leading to the formation of the alternative C3 convertase, C3bBb.

The objective of the present study was to monitor the deposition of complement C3 and IgG from equine serum on yeast cells (Saccharomyces cerevisiae) using a flow cytometric immunoassay. The effects of serum concentration and incubation time were monitored. The opsonic coating was further correlated with the phagocytic capacity using equine blood neutrophils.

MATERIALS AND METHODS

5 x 10^6 heat-killed yeast cells in PBS supplemented with 0.75 mM CaCl_2 and 2.5 mM MgCl_2 (PBS++) were opsonised with pooled adult horse serum at 37ºC.

To block the Mg^{2+}-requiring convertases of both complement pathways, 10 mM ethylene diaminetetraacetic acid was added to non-supplemented PBS (PBS-EDTA) (Fine et al. 1972). Heating of serum to 56ºC for 30 min was also used to inactivate components within both pathways.

To selectively block the Ca^{2+}-dependent classical pathway, 10 mM ethylene glycoltetraacetic acid in PBS with 2.5 mM MgCl_2 was used (PBS-EGTA) (Fine et al. 1972; Kozel 1996). Final serum concentrations ranged from no serum or 0.75–90 %

![Fig 1: Schematic view of pathways for conversion of C3 to C3b.](image-url)
in the studies of the effect of serum dilution, and the incubation time was 15 min. To study the deposition kinetics, 5 or 50 % serum was used and the samples were incubated for 0, 2, 4, 8, 15 or 30 min.

The opsonic coating of C3 and IgG was analysed with a flow cytometric immunoassay. A sheep anti-horse C3 polyclonal antibody (PC261, The Binding Site Ltd, Birmingham, UK) conjugated to a fluorochrome, BODIPY FL-CASE (Molecular Probes, Eugene, Oregon, USA) was used for detection of C3.

To detect IgG, a biotinylated polyclonal rabbit anti-horse IgG antibody (308-065-003, Jackson Immunoresearch Laboratories, Pennsylvania, USA) was followed by streptavidin-R-phycoerythrin (RPE) (R0438; DAKO, Glostrup, Denmark).

Phagocytosis assays were performed as described by Johannisson et al. (1995). The yeast cells were labelled with fluorescein isothiocyanate (FITC) and opsonised with equine serum as described above. EDTA-blood from 3 adult horses was used, after lysis of erythrocytes with a solution containing NH₄Cl. The leucocytes and the opsonised yeast were mixed with a neutrophil:yeast cell ratio of 1:5 and incubated for 15 min at 37ºC for phagocytosis. Fluorescence of extracellular yeast particles was quenched with trypan blue. The percentage of neutrophils with ingested yeast cells was determined with flow cytometry.

**RESULTS AND DISCUSSION**

Opsonic coating of yeast with equine C3 and IgG occurred rapidly. With 50% serum, high C3 deposition was observed as early as at 2 min (Fig 2b). This increased up to 15 min and remained at the same level at 30 min. When only the alternative pathway was allowed to be active by addition of EGTA to 50% serum, or when the serum concentration was low, at 5%, there was a lag period of 15 min before C3 was detected (Fig 2b). The alternative pathway alone could be responsible for high C3-deposition at 30 min when 50% serum was used. However, with only 5% serum, the alternative pathway did not lead to any detectable C3 opsonisation (Fig 2b), even after 30 min.

Substantial amounts of IgG were also deposited within the first minutes of incubation, but the values continued to increase over 30 min when 50% serum was used (Fig 2d).

Inactivation of both complement pathways with EDTA or by heating of serum to 56ºC for 30 min resulted in undetectable levels of deposited C3 regardless of the incubation time or the serum concentration (Figs 2a & b). When complement was inactivated, the fluorescence levels for attached IgG increased 3- to 6-fold, indicating spatial competition between C3 and IgG at binding (Figs 2c,d).
Opsonisation occurred already at low serum concentrations, with detectable levels of deposited C3 and IgG with as little as 0.75% serum.

Complement C3 and IgG deposition increased with the serum concentration, as illustrated in Figs 2a and c. The fluorescence levels indicating IgG deposition increased 15-fold between levels observed with 0.75% serum and 90% serum.

Some results indicated that, normally, there is less IgG than complement C3 on serum-opsonised yeast cells, but quantitative experiments are required to confirm this.

When the classical pathway of complement activation was inhibited with EGTA, there was no complement activation by the alternative pathway when <50% serum was used. However, with 50% serum or more, C3 was deposited also by the alternative pathway, and at a concentration of 90% serum, maximal C3 coating was detected with the alternative pathway alone.

The results from the flow cytometric immunoassay of opsonin deposition correlated well with phagocytosis results (Fig 3). Serum opsonisation was found to be of critical importance for phagocytosis, with no phagocytosis of non-opsonised yeast observed. Complement activation was particularly important at low serum concentrations. When the opsonising serum concentration was low at 1.5%, suboptimal phagocytosis was seen, with recruitment of about half of the neutrophils in phagocytosis. This phagocytic activity was dependent on complement activation by the classical pathway, as there was almost no phagocytosis with the use of EGTA or EDTA.

When the serum concentration was increased to 3%, the percentage of phagocytosing neutrophils was doubled and the mean fluorescence per cell, indicating uptake, was also doubled. Similar magnitudes of phagocytic activity were achieved with further increases of the serum concentration (Fig 3).

A somewhat unexpected finding was that the fluorescence indicating deposition of IgG was enhanced 3-6 fold by complement inactivation. Spatial competition between C3 and IgG may account for this. Thus, more sites for antibody binding may be exposed when complement coating is diminished on the yeast surface. Masking of cell surface antigens by C3b and iC3b has been described (Pangburn 1983). In vivo, turning to more IgG opsonisation in a situation with only a small amount of complement would presumably be beneficial for maintenance of the phagocytic function. However, attempts to evaluate the impact of IgG opsonisation on neutrophil phagocytosis of yeast in the normal situation are not without technical difficulties. If complement activation in serum is inhibited, to separate and study the effect of IgG, more IgG is deposited on the yeast cells, as discussed, compared to opsonisation with unaltered serum. This probably leads to an increased Fc-receptor-mediated phagocytosis in these samples.

It may be concluded that the immunoassay worked well, and opsonisation of yeast particles, leading to phagocytosis, occurs at very low serum concentrations (1.5%). Further, that opsonisation is dependent on activation of the classical complement pathway at this low opsonic level. This is an important finding for efficient host defence, eg extravascular phagocytosis at infection sites.

REFERENCES
FLOW CYTOMETRIC CHARACTERISATION OF NEUTROPHIL PHAGOCYTOSIS AND OXIDATIVE BURST ACTIVITY

S. L. Raidal

Division of Veterinary and Biomedical Sciences, Murdoch University, Perth, Australia

Methods for the evaluation of the functional integrity of phagocytic cells are important tools for investigating the causes of, and host response to, disease. Chemotaxis, phagocytosis (the attachment and internalisation of foreign particles) and the intracellular degradation of ingested material are the principal mechanisms by which phagocytes, such as neutrophils, fulfil their protective role within the body.

Flow cytometry methods have been developed most extensively for the determination of cellular phenotype, using monoclonal antibodies to cell surface antigens coupled with fluorescent markers, and for the evaluation of the nucleic acid content of cells, using DNA or RNA binding dyes. The use of this technology also expedites the evaluation of cell function because it permits rapid evaluation of large numbers of cells on a cell by cell basis, providing greater precision than conventional methods which rely on cell counting or the mean response of a group of cells. Intra- and extracellular events can be discriminated and subpopulations of cells can be identified, negating the need for prior cell separation, which is time consuming and may alter cell function (Bertram and Coignoul 1982; Fearon and Collins 1983; Macey et al. 1992).

PHAGOCYTOSIS

Flow cytometry has been used to evaluate phagocytosis by human neutrophils since the early 1980s and techniques have more recently been described for the evaluation of phagocytic activity of equine neutrophils (Foerster and Wolf 1990; Johannisson et al. 1995; Raidal et al. 1998a). The principle of the phagocytosis assay is illustrated in Fig 1. Neutrophils are discriminated in mixed leucocyte preparations following lysis of erythrocytes, or may be separated by density gradient techniques. Opsonins must be included in assays of peripheral blood neutrophil phagocytosis. Serum (or plasma), purified immunoglobulins or complement may be utilised. Fluorescent labelled particles (bacteria, yeast or latex beads) are added to the cell suspension following pre-opsonisation or the addition of opsonins at the time of assay. Cells which interact with fluorescent particles (by attachment to cell surface membrane receptors and/or by internalisation within the cell) have measurably increased fluorescence and may readily be differentiated from cells which do not have

Fig 1: The principle of flow cytometric phagocytosis assays. Cells are mixed with a source of opsonins for 10 min at 37°C or fluorescent particles (latex beads, bacteria or yeast cells) are opsonised prior to assay. Aliquots of cell suspension are withdrawn for flow cytometric evaluation. Fluorescent particles are added to the cell suspension in an agitating water bath. Cells able to interact with fluorescent bacteria are discriminated (because of their increased fluorescence) from cells unable to do so. Cells which have internalised bacteria are further differentiated by removing the fluorescence of externalised particles by quenching (trypan blue) or counterstaining (eg ethidium bromide).
attached or internalised bacteria. The intensity of fluorescence of positive cells is proportional to the fluorochrome used, the staining intensity of labelled particles, the number of particles interacting with the cell and the amount of protein or DNA degradation occurring within the cell. If a pH sensitive fluorochrome is used, fluorescence intensity is also influenced by the pH of the phagolysosome.

Attached particles can be differentiated from internalised particles by quenching or counterstaining the fluorescence of attached particles, or by the lysis of attached bacterial cells. All methods are dependent on a process which does not change the fluorescence of particles inside the cell membrane, but alters the fluorescence of free or attached particles. Quenching involves the extinction of fluorescence and is achieved most commonly by the addition of trypan blue, which effectively quenches fluorescein isothiocyanate (FITC) (Raidal et al. 1998a) and propidium iodide (Flaminio et al. 1999) fluorescence but is unable to penetrate the cytoplasmic membrane of viable cells. Counterstaining to discriminate attached bacteria may be achieved by using a different colour fluorochrome to change fluorescence by the process of resonant energy transfer (Fattorossi et al. 1989), or by using an antibody to the fluorochrome label derivatised to an alternate fluorescent label (Sveum et al. 1986). Lysis of externalised bacteria has been described in an assay utilising whole blood (White-Owen et al. 1992).

Estimates of the number of attached and/or internalised particles per cell have been reported using direct and indirect methods. Direct methods derive an estimate of the number of particles per cell from the mean fluorescent intensity of the positive cell population. This technique is not suitable when a pH-sensitive dye, such as FITC, is used. Comparison of mean fluorescence of positive cells has been used as an indicator of phagocytic capacity without quantitation of the number of organisms per cell. Refinements of this technique have been described to evaluate protein degradation within the cell, intracellular killing (Bassøe and Bjerknes 1985) and phagolysosome pH (Bassøe et al. 1983a; Rothe and Valet 1988).

Indirect methods for the determination of number of particles per cell are based on subtraction of the number of particles which have not interacted with cells at the completion of the assay from the number of particles initially added to the assay, divided by the number of cells in the assay (Bassøe et al. 1983b; Bjerknes and Bassøe 1984).

Flow cytometric studies of equine neutrophil function have utilised fluorescent microspheres (Foerster and Wolf 1990), bacteria (Johannisson et al. 1995; Raidal et al. 1997; 1998a; 2000; 2001; Flaminio et al. 2000; McTaggart et al. 2001) or yeast particles (Gröndahl et al. 1997, 1999). Commercially prepared latex particles are of uniform size and staining intensity. Phagocytosis assays performed with beads give characteristic multi-peaked positive populations, with each peak corresponding with an integer number of associated particles (Fujikawa-Yamamoto and Odashima 1987; Fredrickson et al. 1992). However, beads are non-physiological and require treatment, such as albumin coating, to aid phagocytosis. Bacterial species utilised in studies of equine neutrophil phagocytosis include Staphylococcus aureus (Raidal 1996; Flaminio et al. 2000; McTaggart et al. 2001), Actinobacillus spp. (Raidal et al. 1997, 1998a, 2000, 2001), E. coli (G. Gröndahl et al. unpublished data) and Streptococcus equi ss. zooepidemicus (Raidal 1996). Fluorescent labelled S. zooepidemicus produced a more heterogeneous fluorescence histogram than other bacterial species, presumably due to variation in chain length (Raidal 1996).

Fluorochromes used for phagocytosis assays bind cellular protein or nucleic acid. FITC and propidium iodide are used most commonly, although Lucifer yellow and ethidium bromide have also been used successfully to label bacteria for the evaluation of phagocytosis by equine neutrophils (Raidal 1996).

Assay conditions, such as the particle-fluorochrome combination, the number of fluorescent particles, staining intensity of particles, source and amount of opsonins and flow cytometer settings must be strictly controlled to achieve reproducible results. An external standard should be passed through the flow cytometer prior to performing repeat assays to ensure machine settings have not altered. The number of neutrophils present in the assay has been shown not to affect the percentage of phagocytosing cells, within the range of 1–12 x 10⁹/l (Hed et al. 1987), although neutrophil numbers are usually adjusted to a more narrow range (5–7 x 10⁹/l). The ideal particle:neutrophil ratio has not been determined. Increased numbers of fluorescent particles are associated with an increased percentage of positive neutrophils and an increased number of particles.
per cell. Low particle:neutrophil ratios may not discriminate functional differences between different cell populations (Miller 1979).

Cells only, or cells plus serum, are used as a negative control, prior to running a phagocytosis assay. An aliquot of cells should be removed immediately after the addition of fluorescent particles (0 min), mixed with an equal volume of PBS with 0.2% w/v EDTA and kept on ice, to arrest phagocytosis. Further aliquots of cells are removed serially during incubation in a 37°C agitating water bath and similarly mixed with PBS-EDTA and placed on ice. Evaluation of the neutrophil population during the assays shows a progressive increase in the percentage of positive cells, from a small number at 0 min (usually less than 5%), to peak usually after 20–30 min of incubation. Extending the assay to 60 min of incubation demonstrates little further increase in the percentage of positive cells (the percentage of positive cells may decrease, presumably associated with reduced fluorescence of internalised bacteria due to increased acidity of the phagolysosome and the degradation of bacterial protein). Mean fluorescence of the positive cell population increases initially (more cells in the brighter fluorescence channels) as the number of bacteria per cell increases, and may decrease after 20–30 min due to internalisation of particles, protein or NA degradation and the more acidic environment within the cells.

**Oxidative burst activity**

The evaluation of neutrophil oxidative burst activity is performed routinely on human neutrophils to identify individuals affected by disorders such as chronic granulomatous disease. The technique has been utilised for the evaluation of equine neutrophil function (Adamson and Slocombe 1995; Raidal et al. 1998b, 2000, 2001; Flaminio et al. 2000; McTaggart et al. 2001). The oxidative (or respiratory) burst is a co-ordinated series of metabolic events initiated when neutrophils are exposed to appropriate stimuli. In addition to the action of neutrophil granule contents, the oxidising agents of the respiratory burst, such as superoxide, peroxide, hydroxyl radical, singlet oxygen and halogenated hydrocarbons, are important for the intra-cellular killing and digestion of microbes.

Flow cytometric methods for the evaluation of oxidative burst are based on the measurement of intracellular oxidative reactions in single cells. The basic principle of most methods is illustrated in Fig 2 and involves loading cells with a non-fluorescent precursor substance which is readily oxidised to a fluorescent compound by components of the oxidative burst. After loading, cells are exposed to a stimulant to induce an oxidative burst response, the magnitude of which is proportional to the fluorescence intensity generated. Most referenced techniques include the addition of azide or cyanide, to inhibit mitochondrial oxidation and hence reduce the amount of oxidation of the substrate prior to the addition of the stimulant (usually referred to as auto-oxidation) and increase the magnitude of the stimulated oxidative burst.

Dihydrorhodamine, hydroethidium and 2',7'-dichlorofluorescin diacetate are the non-fluorescent substrates most commonly used in published techniques. These substrates are oxidised to the fluorescent derivatives rhodamine 123, ethidium and 2',7'-dichlorofluorescein respectively. Hydroethidium is oxidised primarily by the superoxide anion and dichlorofluorescin is oxidised by peroxide (Bass et al. 1983) and...
possibly other reactive oxygen (Rothe and Valet 1990) and nitrogen (Rao et al. 1992) species.

Oxidative burst activity may be initiated by particulate stimuli, such as opsonised zymozan or bacteria, or soluble stimulants, including zymozan activated serum, phorbol esters, a variety of ionophores, bacterial products (Tarigan et al. 1994; Raidal et al. 1998b) and N-formyl methionyl peptides (Lehmeyer et al. 1979). Oxidative activity stimulated by soluble stimulants is independent of phagocytic function and such assays do not require the inclusion of an opsonin. The suitability of phorbol myristate acetate (PMA) as a stimulant of oxidative burst activity in equine neutrophils has been reported (Raidal et al. 1998b). Ethidium bromide (Raidal et al. 1998b) and propidium iodide-labelled bacteria (Flaminio et al. 2000) have also been used to stimulate oxidative burst activity. The magnitude of the response generated was not as great as that elicited by PMA stimulation and peaked after 60 min of incubation. Such techniques have permitted the simultaneous measurement of phagocytosis and oxidative burst activity (Raidal 1996; Smits et al. 1997; Flaminio et al. 2000), although care must be taken to ensure appropriate compensation of spectral overlap between fluorochromes (more difficult in a dynamic assay where fluorescence intensity increases over time), and conclusions on the oxidative capacity of cells must be tempered by awareness that the magnitude of response generated is influenced by the efficiency of phagocytosis (Raidal 1996).

CONCLUSIONS

Flow cytometric techniques are effective tools for the evaluation of non-specific immunity in response to a variety of stressors and for the elucidation of phagocytosis and oxidative burst events within equine neutrophils. The availability of fluorescent markers for the evaluation of calcium flux, other intracellular events and cellular antigen expression offer new opportunities to improve understanding of the mechanisms of health and disease in this species.

ACKNOWLEDGEMENTS

Portions of work completed by the author have been funded by the New South Wales Racing Research Fund and the Rural Industries Research and Development Corporation. The author is supported by the National Australia Bank.

REFERENCES


RT-PCR DETECTION OF EQUINE CYTOKINES

D. W. Horohov

Louisiana State University, Department of Pathobiological Sciences, School of Veterinary Medicine, Baton Rouge, Louisiana 70803, USA

Patterns of cytokine gene expression can be considered characteristic of various protective and pathological conditions (Powrie and Coffman 1993). While the initial detection of cytokines in human medicine and murine models utilised a variety of bioassays, current methods rely almost exclusively on commercially available monoclonal antibodies. Few antibodies are available for equine cytokine detection and researchers must rely instead upon bioassays or mRNA detection techniques to measure cytokine expression in the horse. The latter approach has been facilitated by the cloning and sequencing of a number of equine cytokine genes. A number of methodologies are available for detecting equine cytokine-specific mRNA expression including northern blots, in situ hybridisation (Aggarwal and Holmes 1999), quantitative RT-PCR (Swiderski et al. 1999a), competitive PCR (Giguère and Prescott 1999) and, more recently, ‘real time’ PCR (Leutenegger et al. 1999). While each of these techniques has its unique characteristics, they all share several common features. First, they all rely upon the assumption that cytokines are transcriptionally regulated. Secondly, vagaries in the process of RNA isolation and reverse transcription necessitate the use of internal standards to compensate for yield differences between samples. Lastly, the detection of the product, either directly or following PCR amplification, also varies between the techniques and ultimately requires the determination of copy number within the starting sample.

Protein expression in the cell can be regulated by various mechanisms including transcriptional, translational, post-translational or a combination of all 3 processes. Cytokine gene regulation occurs primarily at the transcriptional level and is mediated by DNA-binding proteins interacting with specific recognition motifs in genetic promoter and enhancer elements (Ye and Young 1997), although interleukin 1 and other inflammatory cytokines are also regulated post transcriptionally (Fenton 1992). As such, detection of mRNA levels for T cell cytokines is considered comparable to detecting the protein itself (O’Garra and Vieira 1992). Nevertheless, issues remain regarding the impact of mRNA half-life and transcriptional rates on RNA-based detection procedures.

Selection of a suitable internal control for RNA detection assays is likewise not without controversy. Since current RNA isolation procedures can result in variations, both in RNA yield and integrity, a ‘housekeeping’ gene is used to correct for inter sample variability. A number of housekeeping genes, including ß-actin (Suzuki et al. 2000), glyceraldehyde-3-phosphate dehydrogenase (GADPH) (Rottman et al. 1996), hypoxanthine phosphoribosyl-transferase (HPRT) (Murphy et al. 1993), and peptidyl-prolyl-cis-trans isomerase (Cyclophilin) (Dozois et al. 1997) have been utilised in the analysis of cytokine mRNA content. One concern regarding any housekeeping gene is the effect of activation state on its expression (Schmittgen and Zakrajsek 2000). Thus, 1.3- to 3-fold increases in ß-actin message have been reported in response to mitogen stimulation using cloned T cells and normal human PBMC (McCairns 1984). Increases in HPRT production in the order of 10–20 fold have been demonstrated in mitogen stimulated human lymphocytes (Steen et al. 1991) making HPRT a less favourable internal control for immunological studies. The primary drawback of ß-actin is that it is a moderately abundant transcript (0.1% of cellular mRNA) whose expression is several orders of magnitude greater than cytokines, a
problem not significantly different for GADPH, cyclophylin and 18s rRNA which are, respectively, moderately abundant, slightly less than moderately abundant transcripts and most abundant (Spencer and Christensen 1999). While each selection has its advocate and detractors (Schmittgen and Zakrajsek 2000), ß-actin was chosen as the internal control in our assays because the equine sequence has been determined allowing the generation of sequence-specific oligonucleotide probes and primers. Further, Swiderski et al. (1999a) have shown that ß-actin is an accurate normalisation factor for inter-sample differences in the RT-PCR assay.

To determine the amount of mRNA in the test samples amplifications from cloned cDNA of identical sequence to the wild type target were used to generate standard curves for the quantification of PCR products (Fig 1). Standard curves are used routinely in a variety of scientific disciplines, including immunology, to evaluate enzymatically dependent phenomena characterised by an exponential rise to maximum. PCR standard curves derived from dilutions of cloned cDNA sequences have been described (Melby et al. 1993). Swiderski et al. (1999a) demonstrated using equine interleukin (IL)-10, that the quantity of mRNA can be determined via RT-PCR using standard curves generated from dilutions of double stranded circular plasmids encoding the transcript of interest; and that, by decreasing the cycle number and cDNA volume used for ß-actin amplification, as compared to cytokine amplifications, ß-actin is an accurate normalisation factor for the differences that are inherent in the RT step of the assay. This approach eliminates the need for synthetic templates common to competitive PCR methods. This method is also less labour intensive because there is no internal standard to construct nor is there a large series of samples with differing competitor:target ratios. Most significantly, this method is performed easily in a single day with very high sample throughput, from cells to quantified product in replicates sufficient for statistical analysis of the data.

This procedure has been utilised to determine cytokine-specific mRNA levels in a variety of samples and model systems including the characterisation of a protective Th2 response to Strongylus vulgaris (Swiderski et al. 1999b), demonstration of a Th1 response in equine recurrent uveitis (Gilger et al. 1999, 2000), and demonstration of a modified Th2 response in summer pasture-associated obstructive pulmonary disease (Horohov 2000). Until monoclonal antibodies to equine cytokines become readily available, we and others shall continue to rely upon these types of procedure to measure cytokine responses in the horse.

REFERENCES


Immunology in 2001

INTRODUCTION

Cytokines are a group of immunologically active peptides which are being applied increasingly in therapeutic and prophylactic regimes in experimental models and in clinical cases. We are interested in the potential application of cytokines as vaccine adjuvants and for the treatment of diseases such as cancer and arthritis in domestic animals. Our current focus in the horse is on cytokines associated with Th1 type immune responses – interferon-γ and interleukins-12 and -18.

EQUINE INTERFERON-γ

Interferon-γ (IFN-γ) has a range of biological activities including activation of macrophages, MHC class I and II induction, promotion of NK cell activity and regulation of other cytokines (Farrar and Schreiber 1993). Equine IFN-γ (eIFN-γ) was cloned by 2 groups independently in 1994 (Curran et al. 1994; Grunig et al. 1994). We have expressed eIFN-γ in bacterial, baculovirus and mammalian systems. Biological activity has been detected by vesicular stomatitis virus cytopathic effect reduction assay for products derived from bacterial, baculovirus and mammalian expression systems (manuscript in preparation). No bioactivity has been associated with bacterial eIFN-γ preparations and it is possible that the purification process inactivates the recombinant cytokine.

Polyclonal antibody preparations raised against bacterial recombinant IFN-γ have been used to derive a sandwich ELISA with a lower detection limit of approximately 100 pg/ml for recombinant eIFN-γ. This has been applied to the analysis of upregulation of IFN-γ in PBMCs incubated with IL-12 preparations (see below).

INTERLEUKIN-12

Interleukin-12 (IL-12) is a key cytokine in the development of Th1 responses through its role in the development of T cells and upregulation of IFN-γ production (Gately et al. 1998). Human IL-12 is a heterodimeric cytokine (p70) formed by p35 and p40 subunits. Expression of p40 in vitro and in vivo is associated with formation of homodimeric p40 molecules which antagonise p70 activity. To circumvent the inhibitory activity associated with p40 homodimer formation, we have adopted the approach of Lieschke et al. (1997) and synthesised equine IL-12 (Nicolson et al. 1999) as a single chain construct with the p35 and p40 subunits covalently linked through a glycine-serine-rich peptide. Expression of single chain equine IL-12 was confirmed by Western blot using an anti-feline p40 peptide serum or a commercially available cross reactive bovine p40 MAb. A bicistronic construct in which the p35 and p40 cDNAs were separated by an IRES element was generated as a source of p70.

Detection of bioactivity of IL-12 expressed in baculovirus and mammalian expression systems was achieved through analysis of IFN-γ induction by equine mononuclear cells. Culture supernatants from cells transfected with single chain or bicistronic IL-12 constructs or infected with baculovirus were positive for IFN-γ induction in this assay. Levels of induction were reduced by prior incubation of supernatants with anti-IL-12 antibody preparations. Human recombinant IL-12
upregulated IFN-γ production in this assay. Bioactivity of baculovirus-expressed eIL-12 was also verified using a proliferation assay in which infected culture supernatants were incubated with equine PBMCs pre-incubated with PHA and IL-2 (McMonagle et al. 2001).

**INTERLEUKIN-18**

Interleukin-18 (IL-18) is a potent inducer of IFN-γ and acts synergistically with IL-12 in this respect (reviewed in Dinarello 2000). IL-18 is synthesised as an inactive precursor which requires cleavage of an N-terminal peptide by caspase-1 for release of bioactive mature IL-18. Wardlow et al. (1999) cloned equine caspase-1 cDNA and are currently investigating secretion and bioactivity associated with several forms of equine IL-18 constructs in vitro.

A set of monoclonal antibodies to equine IL-18 have been recovered. Based on cross reactivity with feline IL-18, it was surmised that a minimum of 2 epitopes are recognised. A sandwich ELISA has been developed with a sensitivity of approximately 2 ng/ml for detection of bacterially generated recombinant equine IL-18.

Human myelomonocytic KG-1 cells are inducible for IFN-γ production on incubation with human and murine IL-18, although the sensitivity of detection of the latter is reduced 100-fold (Konishi et al. 1997). Incubation of cell lysates harvested from equine proIL-18 transfected cells with human recombinant caspase-1 resulted in detection of an IL-18 protein of a size consistent with that of predicted mature IL-18. Incubation of this material with KG-1 cells was associated with induction of human IFN-γ as assessed using a commercial human IFN-γ ELISA. This is suggestive of equine IL-18 bioactivity in the KG-1 assay although the sensitivity relative to that of human IL-18 is unknown. Harvests from cells transfected with IL-18 constructs with an artificial signal peptide were also associated with induction of IFN-γ by these cells. Preliminary data indicate that the level of IFN-γ induction can be diminished through incubation of cell lysates with anti-IL-18 monoclonal or polyclonal antibody preparations prior to application to KG-1 cells.

**THE FUTURE**

We now have a complement of equine-specific cytokine and serological reagents and have verified bioactivity of equine IFN-γ, IL-12 and IL-18. The next step will be the assessment of these cytokines as vaccine adjuvants in the horse.

**REFERENCES**


Cytokines are hormone-like low molecular weight proteins produced by various types of somatic cells in response to a number of inducing stimuli. Cytokines regulate both the initiation and maintenance of immune and inflammatory responses. They also play a role in many important biological functions such as embryological growth and development, stem cell growth and differentiation, tissue repair, endocrine functions, aging, etc. Over the last 10 years, classification of murine and, to a lesser extent, human T lymphocytes into type 1 and type 2 subsets has provided a useful framework for understanding immune response in infectious diseases (Mossman and Sad 1996). Although it remains to be definitively established whether this paradigm can be applied to the horse, preliminary studies suggest the presence of type 1- and type 2-like cells in equine long-term T-cell culture (Aggarwal and Holmes 1999). Knowledge of the equine immune system and understanding of the pathophysiology of inflammatory and infectious diseases of the horse and other domestic animal species has been limited in part by the lack of species-specific reagents available for measuring cytokines. Some progress has been made by adapting bioassays for only a few cytokines that function in a non-species-specific manner (Seethanathan et al. 1990; Morris and Moore 1991; Mackay and Lester 1992). However, such assays are cumbersome and have low specificity, and some equine cytokines such as IL-2 and IL-4 do not exert activity on murine cells (Dohmann et al. 2000). The development of immunoassays in the horse has been limited by the lack of species-specific monoclonal antibodies and cytokine standards. Because of this limitation with bioassays and immunoassays in the horse, and because of the apparent correlation between cytokine gene expression and protein production (Cherwinski et al. 1997), several researchers have relied on detection of equine cytokine mRNA expression. Thus the cloning, sequencing and expression of a number of equine cytokines have been accomplished (Curran et al. 1994; Grunig et al. 1994; Kato et al. 1997; Kato et al. 1995; Penha-Goncalves et al. 1997; Su et al. 1991; Nicolson et al. 1999). Equine-specific genomic or cDNA sequences currently available in GenBank include IL-1α, IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p35, IL-12 p40, IL-18, IFN-α, IFN-β, IFN-ω, IFNγ, TNF-α, and TGF-β.

Several techniques are available to detect mRNA expression. Because it is rapid and considerably more sensitive than traditional RNA blot techniques, RT-PCR is increasingly being used to detect small changes in gene expression that would otherwise be undetectable. Although RT-PCR offers many advantages over RNA blot methods, it can be difficult to obtain quantitative information. This is mainly because of the exponential nature of PCR, where small tube-to-tube variation in amplification efficiency have dramatic effects on product yield. Furthermore, the amount of PCR product generated reaches a plateau after several cycles of amplification through consumption of necessary components and the generation of inhibitors, obscuring differences in the initial amount of target DNA (Gause and Adamovicz 1995). Truly quantitative methods are required for accurate comparison of cytokine expression between groups of animals or in response to treatment or vaccination. A quantitative RT-competitive PCR (RT-cPCR) assay for the measurement of equine cytokines has been developed (Giguère and Prescott 1999). This assay proved accurate and reproducible for quantitation of mRNA expression for cytokines expressed at a
high level. However, quantitation of mRNA expression for cytokines expressed at low levels such as IL-4 and IL-5 is difficult or sometimes impossible using RT-cPCR (Giguère et al. 1999). Also, RT-cPCR requires testing several dilutions of a competitor DNA or RNA fragment along with the sample. This is labor intensive and the amount of sample used for each dilution limits the number of cytokines that can be measured and prevents replicate determination of the same samples. Furthermore, RT-cPCR entails laborious post PCR processing steps for quantitation of target and competitor fragments. Extensive manipulation of post PCR products is also a significant source for contamination of pre-PCR samples.

The ability to monitor the real-time progress of PCR has completely revolutionised quantitation of DNA and RNA. Real-time PCR detects specific nucleic acid amplification products as they accumulate by using a fluorogenic oligonucleotide probe. Attached to the hybridisation probe are a reporter fluorescent dye and a quencher dye. In an intact probe, emission from the reporter dye is minimal due to the energy transfer to the quenching dye and negligible fluorescence is observed. During PCR amplification, as the newly synthesised DNA strand reaches the site of probe hybridisation, the DNA polymerase cleaves the probe, releasing the reporter dye from the quencher dye. The fluorescence intensity therefore increases proportionally to the amount of DNA amplified. The fluorescence signal is detected in all 96 wells during every cycle of amplification. Detection of fluorescence by real-time PCR is several fold more sensitive than conventional analysis of PCR products on agarose gel (Lockey et al. 1998). The high sensitivity of this technique allows the use of less sample material. Because detection of the signal depends on hybridisation of a gene-specific probe, real-time PCR is also more specific than traditional PCR systems. Rather than monitoring final amplification of product as done in traditional PCR, the real-time PCR reactions are characterised by the point in time when amplification is first detected rather than the amount of PCR product accumulated after a fixed number of cycles. This point is referred to as threshold cycle (Ct). Detection of the Ct ensures quantitation during the exponential phase of PCR, therefore avoiding problems related to detection of PCR products after the plateau has been reached.

Real-time PCR eliminates the need for a competitive fragment to be amplified with the target allowing the measurement of more samples on the 96-well plate. Real-time quantitation eliminates post-PCR processing of PCR products, which not only increases throughout and reduces the chances for carry-over contamination, but also removes post PCR processing as a potential source of error. Besides quantitation of mRNA expression for cytokines or other proteins, real-time PCR can also be used for detection of microorganisms, quantitation of viral load, gene knockout analysis and single nucleotide polymorphism screening.

**REFERENCES**


Immunopathol. 48, 221-231.


Influenza viruses infect many species including birds, swine, man and horses in which the virus is an important respiratory pathogen. In most microbial infections, the early immune and inflammatory responses are crucial not only for elimination of infectious agents but also for severity of clinical signs. In human, murine and porcine models of influenza, the virus induces production of a number of proinflammatory cytokines such as type I interferons (IFNs), tumour necrosis factor-α, interleukin-1 (IL-1), IL-6, IL-8 and other monocyte-attracting chemokines (Julkunen *et al.* 2001; Van Reeth 2000). Many of these have wide ranging effects, eg type I IFNs have direct and indirect antiviral effects, induce pyrexia and are important in the regulation of ensuing immune responses towards a Th1 phenotype (Bogdan 2000). In horses, very few data exist on cytokine production in response to influenza infections, but IFN activity has been detected in nasal secretions during a natural influenza outbreak (Jensen-Waern *et al.* 1998). The present study aimed to monitor production of type I IFN and IL-6 during an equine influenza A2 virus infection in the natural host. The effects of 2 ‘European-type’ influenza strains, Newmarket/2/93 and Sussex/89, were compared. The latter is considered to be the more pathogenic.

Twenty Welsh mountain ponies, seronegative to influenza by SRH, were infected with equine influenza A2 by nebulised aerosol on Day 0. Group A (n=10); were infected with Sussex/89 and Group B (n=10) with Newmarket/2/93. Virus excretion and clinical signs were monitored and rectal temperatures recorded daily until Day 10. Serum samples were collected for cytokine determination on Days 0–10 and nasal secretions sampled (with plain cotton buds) on Days 1–10. IFN activity was detected with a VSV-inhibition bioassay on MDBK cells. IL-6 activity was determined with the IL-6 dependent murine cell-line B9.

Virus was isolated from nasal swabs from all experimentally infected ponies, with maximum excretion on Days 2 and 3. There was no difference between the 2 groups in the onset of virus excretion or the amount of virus excreted. Group A tended to excrete virus for a longer period (until Day 7 compared to Day 6 in Group B). The experimental infection induced coughing and nasal discharge from Day 2 in both groups. However, throughout the experiment, more ponies coughed in Group A than Group B. Onset of nasal discharge was earlier in Group A, where more ponies displayed discharge in the initial phase of the experimental infection (until Day 6). In the second week of infection there was no difference in the number of ponies with nasal discharge in the 2 groups. Increased rectal temperature was seen post infection in both groups. All Group A ponies had pyrexia (≥ 38.9°C) on Day 2 and/or Day 3. In Group B, 6 ponies had pyrexia on Day 2. On average, Group A ponies were pyrexic from Days 2 to 4 inclusive, whereas Group B ponies were pyrexic on Day 2 only.

IFN was detected in nasal secretions from all ponies in Group A on at least one sampling occasion between Days 2 and 6 (Table 1). In contrast, only 2 ponies in Group B displayed IFN in nasal secretions. No IFN was detected in serum samples collected throughout the study.

IL-6 activity was detected in nasal secretions from all experimental ponies. Ponies infected with Sussex/89, however, showed a markedly higher response with peak levels on Day 3. Newmarket/2/93-infected ponies showed low but detectable levels from Day 2 onwards (Fig 1).

In serum, Group A showed a low IL-6 response from Day 1, whereas Group B displayed occasional IL-6 positive serum samples only (Table 2).

Taken together, both equine influenza A2 infections elicited local IFN and IL-6 responses. In
TABLE 1: No. of ponies with IFN in nasal secretions (Positives), and range of IFN levels after experimental infection with equine influenza A2 Sussex/89 (n=10) or Newmarket/2/93 (n=10) on Day 0

<table>
<thead>
<tr>
<th>Day</th>
<th>Sussex/89</th>
<th>Newmarket/2/93</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positives</td>
<td>Positives</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Day 2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Day 3</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Day 4</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Day 5</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Day 6</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Day 7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Day 8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Day 9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Day 10</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

TABLE 2: No. of ponies with IL-6 in serum (positives), and range of IL-6 levels after experimental infection with equine influenza A2, strain Sussex/89 (n=10) or Newmarket/2/93 (n=10) on Day 0

<table>
<thead>
<tr>
<th>Day</th>
<th>Sussex/89</th>
<th>Newmarket/2/93</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positives</td>
<td>Positives</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Day 2</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Day 3</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Day 4</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Day 5</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Day 6</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Day 7</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Day 8</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Day 9</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Day 10</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

the case of IL-6, low systemic levels of the cytokine were also observed, probably due to ‘leakage’ into the circulation and a reflection of the local IL-6 production. Infection with Sussex/89 induced IFN production in all ponies and markedly higher IL-6 responses compared to Newmarket/2/93. Whether these differences in cytokine production are due to strain differences in cytokine inducing capacity or secondary to other viral virulence factors needs to be addressed. Nevertheless, the different cytokine production induced by the 2 strains may contribute to the difference in pathogenicity. Further, the marked production of IL-6 in nasal secretions emphasises the importance of this cytokine in regulating and stimulating local antibody responses to mucosal infections. This supports the potential of exogenous IL-6 (eg, as DNA or recombinant protein) to enhance local immune responses to mucosally-administered vaccines.

ACKNOWLEDGEMENTS

This study was supported by the Swedish Council for Forestry and Agricultural Research and the Linnéa and Axel Ericsson Fund. We thank Sarah Flatt and Zoe Swann for excellent technical assistance and Caroline Fossum for discussion and scientific support.

REFERENCES


Our goals are to develop practical strategies to prevent the establishment of infection in equine fractures contaminated with bacteria and to eradicate orthopaedic infection quickly and economically. We believe the inability of the local immune system to control infection is the primary reason for treatment failure. We have developed an in vivo model of porcine implant-associated orthopaedic infection with *Staphylococcus aureus* to analyse the genomics of the local immune response using DNA microarrays.

Microarrays represent high throughput technology to determine differential gene expression. They are an important experimental approach to identifying and interpreting changing levels of individual transcripts in various biological systems. Several thousand discrete DNA sequences (known genes or expressed sequence tags) can be printed (arrayed) on a glass slide using an arrayer robot. Messenger RNA is isolated from cell populations from 2 tissues of interest and labelled with fluorescent dyes (typically red and green). The labelled samples are mixed and hybridised (allowed to bind to sequence complements) to the arrayed DNA spots. Using a scanner, the ratio of colours is measured, giving a comparative analysis of the relative abundance of transcripts in both samples. This allows simultaneous, rapid monitoring of many differentially expressed genes. Although establishment of the procedure initially requires a substantial investment, processing of individual samples is relatively inexpensive.

DNA microarrays have been used to explore the genomes of several model organisms (3-15,000 genes) and provide vital information about the relationship between gene expression and the subsequent effect of the gene product on the physiology of the organism. It is believed that further use of DNA microarrays will generate crucial information about gene function in more complex organisms by identifying and analysing patterns of co-expression and degree of sequence similarity (identification of orthologous genes).

Microarrays have been used in man to investigate disease-related gene activity and to profile diseases. In orthopaedic infection, inflammation is a complex process caused by many different cell types in the bone, surrounding tissues and circulation. Surveying gene expression in cells from these sites will provide insight to the development of pathology (absolutely and temporally), and an opportunity to identify target genes for treatment intervention. We anticipate a differential expression of genes between non-infected and infected fractures. Monitoring local and systemic immune responses will elucidate the immune response to orthopaedic implants and infection and could identify host genetic markers for infection that facilitate diagnosis of implant-associated orthopaedic infection.

We have cloned and sequenced 10,000 ESTs from 5 immune tissues (lymph node, spleen, peripheral blood cells, thymus and bone marrow) in pigs with implant associated orthopaedic infection. Annotated transcripts and ESTs were used to construct a cDNA microarray. This resulted in the construction of microarrays, identification and radiation hybrid mapping of the proeasome/ubiquitin system and a first generation EST radiation hybrid map of the porcine genome.

Our work with pigs and horses is ongoing and we will evaluate the use of heterologous cDNA microarrays to monitor gene expression in equine orthopaedic infection using peripheral blood cells, the cell population accessible with minimal invasion. We also hope to construct an equine DNA microarray to evaluate the specific genes transcribed during equine orthopaedic infection.
THE POWER OF LIMITING DILUTION ANALYSIS

J. H. Kydd, E. Wattrang*, G. P. Allen†, T. O’Neill and D. Hannant

Animal Health Trust, Lanwades Park, Kentford, Newmarket, Suffolk CB8 7UU, UK; *Unit for Comparative Medicine & Physiology, Department of Large Animal Clinical Sciences, PO Box 7018, SE-750-07, Uppsala, Sweden; †Gluck Equine Research Centre, Department of Veterinary Science, University of Kentucky, Lexington, Kentucky 40546-0099, USA

Limiting dilution analysis (LDA) can be used to quantitate the in vitro activity of a responding cell type within a mixed leucocyte population. Using a linear concentration range, aliquots of cells are cultured in microwells under optimum conditions so that an individual immune cell can be amplified into a measurable clone of cells and the well scored for the presence or absence of a precursor/memory cell. Large numbers of replicates (at least 24 wells) are required and the results are reported as the fraction of negative wells. The negative log of the fraction of non-responding cultures is linearly proportional to the mean number of precursor cells per culture with the experimental plot passing through the origin. By interpolating the intercept of this line with 37% non-responding cultures, the frequency of precursor cells can be estimated. The preferred statistical method to calculate the frequency is currently the Jacknife version of Maximum Likelihood. LDA can be applied to any randomly distributed cellular or subcellular system and provides a longitudinal measure of events within the individual. To date, lymphocyte frequencies that have been successfully investigated using LDA in several species include antigen specific B or T cells, cytotoxic lymphocyte precursors (CTLp) and cytokine producing cells.

CTL frequencies were determined in horses in an effort to identify a correlate of immunity to equine herpesvirus-1 (EHV-1). The working hypothesis was that high frequencies of EHV-1 specific CTL correlate with reduced clinical and virological signs after experimental infection and furthermore protect pregnant mares from virus induced abortion. A total of 25 ponies in 5 groups were used to test this hypothesis (Table 1). ‘Primed’ ponies had received no experimental infections but were assumed to have had at least one natural field infection with EHV-1. ‘Multiply infected’ ponies had received at least 3 experimental infections with EHV-1 (strain Ab4/8) on an annual basis (O’Neill et al. 1999 for details). Groups 1 and 2 were adult non-pregnant animals and Groups 3, 4 and 5 consisted of pregnant mares. The Group identities were as follows: Group 1: 5 primed ponies; Group 2: 3 multiply infected ponies; Group 3: 5 primed pregnant mares; Group

### TABLE 1: Circulating EHV-1 specific CTL frequencies before and after challenge infection in 5 groups of ponies

<table>
<thead>
<tr>
<th>Group</th>
<th>Status</th>
<th>Mean ± se CTL frequency/10⁶ PBMC</th>
<th>Outcome of pregnancy</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre-infection</td>
<td>Post vaccination</td>
<td>Post infection*</td>
</tr>
<tr>
<td>1</td>
<td>Primed</td>
<td>0.5±0.6</td>
<td>-</td>
<td>4.1±6.2</td>
</tr>
<tr>
<td>2</td>
<td>Multiply infected</td>
<td>45.3±40.0</td>
<td>-</td>
<td>32.4±34.3</td>
</tr>
<tr>
<td>3</td>
<td>Primed, pregnant</td>
<td>ud</td>
<td>-</td>
<td>1.4±1.0</td>
</tr>
<tr>
<td>4</td>
<td>Primed, pregnant</td>
<td>2.7±3.6</td>
<td>2.0±2.6</td>
<td>5.7±5.5</td>
</tr>
<tr>
<td>4</td>
<td>Primed, pregnant</td>
<td>0.9±1.4</td>
<td>0.3±0.2</td>
<td>2.5±2.1</td>
</tr>
<tr>
<td>5</td>
<td>Multiply infected, pregnant</td>
<td>4.7±0.6</td>
<td>-</td>
<td>5.0±2.6</td>
</tr>
</tbody>
</table>

*Post infection samples: Groups 2 & 3 sampled at 3 weeks post infection, Groups 4 & 5 sampled at 7 weeks post infection; na = not applicable; ud = undetectable
4: 9 pregnant mares divided into 5 vaccinates (inactivated EHV-1 in carbomer adjuvant vaccinated im at 5, 7 and 9 months of gestation) and 4 controls; Group 5: 3 multiply infected pregnant mares. All ponies were infected intranasally with Ab4/8, a virulent strain of EHV-1, then monitored over a 2 week period for clinical, serological and virological signs of infection. Group 4 mares were challenged 4 weeks after V3. Nasopharyngeal swabs and peripheral sera and mononuclear cells (PBMC) were collected from all ponies before infection, after vaccination but before infection (Group 4 only) and following infection. Seroconversion of complement fixing antibodies, pyrexia, nasopharyngeal virus shedding and cell associated viraemia occurred in all ponies except the immune Group 2 animals and Group 5 pregnant mares (see below).

CTL were induced from PBMC in the presence of Mitomycin C-treated PBMC as antigen presenting cells, infectious virus and immune equine serum in 96 well plates for 7 days. Wells were then split 3 ways and different radioisotopically labelled target cells added. Target cells comprised autologous or heterologous EHV-1 infected pokeweed mitogen lymphoblasts or mock infected autologous lymphoblasts. Following a 4 h incubation, supernatants were harvested and gamma emissions counted. Previous data show that the cytotoxic activity measured in this assay is MHC class I restricted, specific for EHV-1 and mediated by CD8+ lymphocytes (O’Neill et al. 1999). The results (Table 1) suggest that frequencies of EHV-1 specific CTL are lower in primed but susceptible animals (Group 1) which exhibit the normal clinical and virological signs of disease after experimental intranasal infection with Ab4/8 (O’Neill et al. 1999) than CTL frequencies in multiply infected, immune ponies (Group 2).

Three groups of pregnant mares were infected with EHV-1 and their frequencies of CTL measured. The 5 primed mares in Group 3 all had undetectable levels of CTL prior to experimental infection and all aborted. For these mares, CTL frequencies increased 3 weeks after infection. Group 4 control mares had a low mean CTL frequency immediately before infection and all aborted but frequencies increased at 7 weeks after infection. Vaccinates in Group 4 had a higher CTL frequency than controls both before and after vaccination and the mean frequency also increased at the 7 week sampling point. Of 5 vaccinated mares, 4 foaled. The Group 4 vaccinated mares had higher CTL frequencies before vaccination and challenge infection than the controls although the difference was not significant. There was no evidence that vaccination with inactivated virus increased the CTL frequency. Group 5 contained 3 multiply infected mares which had higher frequencies of CTL before infection and these CTL numbers were maintained up to the 7 week sampling point after infection. All 3 of these mares foaled. Neither clinical, serological nor virological signs of infection could be detected among Group 5 animals.

Comparison of foaling vs aborting mares using Students t test illustrated that successful foaling correlated directly with significantly higher frequencies of CTL both before (P<0.01) and after (P<0.01) infection. The duration of nasal virus shedding was also significantly shorter (P<0.02) in mares which foaled. There was no significant difference in duration of cell associated viraemia between the foaling and aborting groups.

These results suggest that a high frequency of EHV-1 specific CTL (precursors and memory cells) before infection correlates directly with reduced nasal shedding of infectious virus and successful foaling in pregnant mares. In conclusion, LDA is a powerful technique, applicable to many systems and it has enabled the identification of a potential correlate of immunity to EHV-1 infection in horses.

ACKNOWLEDGEMENTS

The financial assistance of the Equine Virology Research Foundation, Horserace Betting Levy Board and Fogarty International Research Fellowship is gratefully acknowledged.

REFERENCES

FLOW CYTOMETRIC TECHNIQUES IN CLINICAL INVESTIGATION

B. R. Rush, M. J. B. F. Flaminio*, E. G. Davis and M. J. Wilkerson

College of Veterinary Medicine, Kansas State University, Kansas; *The James A. Baker Institute for Animal Health, Cornell University, Ithaca, New York 14853, USA

Flow cytometric analysis has become a valuable tool for clinical investigation of immunosuppressive, immune-mediated and neoplastic disease in horses. Flow cytometry provides rapid assessment of proportions of cellular populations and, in some instances, can evaluate cellular function. Clinical application of flow cytometric analysis is relatively common in human medicine and is used predominantly for evaluation of immunosuppressive and neoplastic disorders.

Flow cytometric determination of lymphocyte subpopulations allows the equine clinician to detect immunosuppressive disorders, monitor the response to immunostimulant therapy and characterise lymphoid neoplasias. In human medicine, determination of CD4+ and CD8+ lymphocytes is performed routinely in patients with HIV to monitor disease progression and direct prophylactic therapy. Lymphoid neoplasias are characterised to predict the biological activity of the tumour.

Phagocytosis and oxidative burst activity of equine leucocytes may be determined simultaneously via flow cytometry. In this technique, propidium iodide (PI)-labelled Staphylococcus aureus is used to measure uptake of bacteria by equine phagocytes and oxidative burst activity is measured by oxidation of dehydrododarine 123. The advantage of the simultaneous technique is to provide independent and combined assessment of phagocytosis and oxidative burst. In addition, this assay allows the use of autologous heat-inactivated sera to evaluate opsoniation capacity of antibody separately. Application of this technique includes identification of inherited neutrophil function disorders, prognosis and treatment of septicaemia, and effects of exercise and long distance transport on phagocyte function.

Immune-mediated hemolytic anaemia can be diagnosed using flow cytometric direct immunofluorescent assay. This rapid, quantitative assay is more sensitive than the Coombs’ test for detection of membrane-bound antibody and can identify the isotype-specific immunoglobulin (IgG, IgA, IgM). IgG is the most common isotype, although IgM and IgA have been implicated in clinical cases of immune-mediated haemolytic anaemia in horses. The DIF technique can detect small numbers of immunoglobulin-bound red blood cells and is independent of the prozone effect. Serial direct immunofluorescent assay allows the equine clinician to monitor response to immunosuppressive therapy.

The flow cytometric direct immunofluorescent assay has been inconsistent for diagnosis of immune-mediated thrombocytopenia in horses. The technique has been reliable in select horses, but has produced apparent false negative and false positive results in others. Flow cytometry has been valuable for detection of reticulated (regenerative) platelets in horses with thrombocytopenia using thiazole orange. Thiazole orange permeates the cell membrane, binds nucleic acids and emits fluorescence detected by the flow cytometer. Immature platelets contain increased amounts of nucleic acids, predominantly mRNA, which is detected by the flow cytometer. The technique is a simple, non-invasive alternative to bone marrow aspirate/biopsy for characterisation of thrombocytopenia in horses as regenerative vs non-regenerative.

In human medicine, DNA cell cycle analysis via flow cytometry is used to characterise neoplastic and metaplastic tissues and predict their biological behaviour. In some cases, cell cycle analysis provides more powerful prognostic information than any other parameter. Cell cycle
analysis evaluates the cellular population. Propidium iodide binds to cellular DNA at all stages of the cell division cycle, and the intensity of PI emission is directly proportional to its DNA content. Cells in G0 and G1 have a diploid chromosomal number (32 pairs), and dividing cells (G2 and M-phase) have double DNA content (tetraploid). During DNA synthesis (S-phase), the DNA content increases progressively until tetraploidy is achieved. Single colour flow cytometric analysis produces a DNA histogram, and the major fluorescent intensity peaks correspond to specific phases of the cell cycle. Many malignancies have abnormal chromosomal content (aneuploidy), which will appear as an aberrant peak in the cell cycle histogram. The aggressive nature of a tumour is evaluated by determining the percentage of cells in S-phase and documenting aneuploidy. In human medicine, these flow cytometric parameters are used to determine prognosis (5 year survival) and assist with the development of a treatment plan. Cell cycle analysis can predict the likelihood of response to specific therapies (radiation, endocrine, chemotherapy) and disappearance of aneuploidy indicates a favourable response to treatment.

In veterinary medicine, cell cycle analysis has been evaluated to differentiate inflammatory/granulomatous conditions from equine and canine lymphoma. Peripheral blood, body cavity effusion, fine needle aspirate and biopsy samples can be evaluated via cell cycle analysis. Formalin-fixed samples can be evaluated but paraffin-embedded samples are more difficult to process and interpret. Cell cycle analysis is more sensitive than cytological evaluation for detection of neoplastic cells in effusions and peripheral blood, although not all equine lymphoma patients have circulating neoplastic lymphoblasts or exfoliative neoplastic cells in malignant effusion. Therefore, false negative findings are common with peripheral blood and effusive samples in horses. Cell cycle analysis of fine-needle aspirate and biopsy samples of equine lymphoma have demonstrated marked aneuploidy (hyperploidy) and increased S-phase activity. Most human lymphomas demonstrated aneuploidy, primarily hyperploidy, although some are diploid. Occasionally, hypoploidic human and canine lymphomas are observed. The majority of canine lymphomas are diploid but approximately 25% of canine lymphomas demonstrate aneuploidy (hyperploidy). All human and canine lymphomas have increased S-phase activity. Our laboratory has not identified any diploid equine lymphosarcomas. Normal values for cell cycle analysis of equine lymph nodes have been determined in our laboratory. Reactive (inflammatory) lymph nodes demonstrate a moderate increase in S-phase activity with minimal to no aneuploidy. DNA cell cycle analysis of effusion, tissue and blood provides a rapid indication of the likelihood of malignancy in horses. The technique has proved valuable in differentiation of neoplastic conditions from inflammatory and granulomatous masses. Identification of occult neoplasias using peripheral blood or effusion requires the presence of neoplastic cells in the sample; negative findings should not be interpreted as definitive evidence of an inflammatory process.
Based on previously reported flow cytometric methods for the analysis of phagocytic function in human, bovine and equine phagocytes (Hasui et al. 1989; Perticarari et al. 1991; Smits et al. 1997; Raidal et al. 1998a,b), a simple, rapid and precise methodology was developed to evaluate simultaneously phagocytosis and oxidative burst activity (OBA) in horse cells. The technique has been described by Flaminio et al. (2000, 2001). Briefly, propidium iodide-labelled Staphylococcus aureus (PI-Sa) is used to measure uptake of bacteria by equine phagocytes. Trypan blue is added to quench fluorescent signals imparted by non-phagocytised bacteria or extracellular bacteria that adhere non-specifically to the surface of the phagocytes. Trypan blue is added to quench fluorescent signals imparted by non-phagocytised bacteria or extracellular bacteria that adhere non-specifically to the surface of the phagocytes. Trypan blue is added to quench fluorescent signals imparted by non-phagocytised bacteria or extracellular bacteria that adhere non-specifically to the surface of the phagocytes. Trypan blue is added to quench fluorescent signals imparted by non-phagocytised bacteria or extracellular bacteria that adhere non-specifically to the surface of the phagocytes. Trypan blue is added to quench fluorescent signals imparted by non-phagocytised bacteria or extracellular bacteria that adhere non-specifically to the surface of the phagocytes. Trypan blue is added to quench fluorescent signals imparted by non-phagocytised bacteria or extracellular bacteria that adhere non-specifically to the surface of the phagocytes. Trypan blue is added to quench fluorescent signals imparted by non-phagocytised bacteria or extracellular bacteria that adhere non-specifically to the surface of the phagocytes.

Oxidation of non-fluorescent dihydrorhodamine 123 (DHR) by reactive oxygen intermediates to the green fluorescent rhodamine 123 (RHO) is measured after phagocytosis of bacteria. The log red (PI, FL2) fluorescence is proportional to the number of ingested bacteria. The log green (RHO, FL1) fluorescence is proportional to the OBA activity. In stimulated cells, fluorescence imparted by the conversion of DHR to RHO is proportional to the level of activation of phagocytes following ingestion of opsonised and non-opsonised bacteria.

Opsonised reactions have greater mean fluorescence values than non-opsonised reactions (Bassøe and Bjerknes 1984). Phagocytic activity is improved by the presence of opsonins, which may function via C3b receptors (for complement) or Fc-receptors (for immunoglobulins). This activity is dependent on the amount of serum added to the bacteria, because opsonisation with 10% horse serum results in lower phagocytic and OBA compared to 20%, 30% and 40% serum (Flaminio et al. 2000). In addition, phagocytosis activity is reduced when heat-labile opsonins (which include complement) are inactivated (Bassøe and Bjerknes 1984). Immunoglobulins and complement reveal synergistic interaction in phagocytosis and oxidative burst activity. One of the advantages of this assay is the possibility of incubation of leucocytes with a standard source of pooled sera for opsonisation, which reduces the variability in phagocytic response caused by individual differences in opsonic activity (Grøndahl et al. 1997). In addition to evaluating individual phagocytosis and cellular oxidative activity, this technique allows the use of autologous and heat-inactivated sera to characterise opsonisation capacity (complement x antibody).

Foal neutrophil function is dependent on the maturation of humoral opsonic factors rather than on maturity of neutrophil function (Grøndahl et al. 1999; Flaminio et al. 2000). For this reason, foals may have decreased neutrophil activity in vivo in the first few months of age compared to adult horses. This effect becomes more evident by the benefits of plasma therapy in neonates, which...
significantly increases opsonic capacity of foals’ sera (Gröndahl et al. 1999).

The application of this flow cytometric technique allowed the evaluation of phagocytosis and oxidative burst activity in foals, as well as the opsonic capacity of their serum. Blood samples from 10 foals (6 females, 4 males) of mixed breeds were collected in heparinised tubes and tubes with no additives at 24 h (post colostrum ingestion), weekly for the first month of life and monthly up to 4 months of age. All foals in this group had adequate transfer of maternal immunoglobulins. The results demonstrate no age-related differences in the phagocytic capacity of foal neutrophils when adult serum was used for opsonisation, indicating that equine neonate phagocytes are competent (Fig 1). However, adult horse phagocytes had inferior function when sera from foals of different ages were used for opsonisation. Moreover, normal foal serum showed age-related improvement of opsonic activity, whereas heat-inactivated serum did not show changes (Fig 2).

Simultaneous analysis of phagocytosis and oxidative burst activity by flow cytometry is a rapid and sensitive method to evaluate phagocyte function in horses. Potential applications of this technique include identification of inherited neutrophil function disorders, prognosis and treatment evaluation of septicemia, and effects of exercise and transportation on phagocyte function.

REFERENCES


SESSION III:

Inflammation

Chairman: Paul Lunn
CHRONIC AIRWAY DISEASE IN THE HORSE

N. E. Robinson

Department of Large Animal Clinical Sciences, Michigan State University, East Lansing, Michigan 48823, USA

Chronic airway disease in the horse is also known as equine chronic obstructive pulmonary disease (COPD). The latter name is inappropriate because human COPD is largely a disease of smokers and is generally unresponsive to therapy with corticosteroids or bronchodilators. By contrast, equine chronic airway disease is very responsive to these modes of treatment. A recent workshop (Robinson 2001) recommended that 2 phenotypes of equine chronic airway disease be recognised, heaves or recurrent airway obstruction (RAO) in middle-aged horses and inflammatory airway disease (IAD) in young performance horses in training. RAO is a severe inflammatory obstructive airway disease of middle-aged and older horses (Robinson et al. 1996). It is induced by exposure of susceptible horses to inhaled organic dust, generally from hay. A similar syndrome, summer pasture-associated obstructive pulmonary disease (SPAOPD) occurs in the southern United States during the summer months (Seahorn and Beadle 1993) and is believed to be due to inhalation of spores from molds that grow in lush pasture. When horses feed, they push their noses into the hay and other feeds and thereby expose themselves to high levels of dust in their breathing zone (Woods et al. 1993). Hay dust is a mixture of mold spores, forage mites, particulates and endotoxins (Woods et al. 1993; Vandenput et al. 1997; McGorum et al. 1998). All of these agents can either induce or exacerbate airway inflammation. When dust exposure is reduced either by returning the horse to pasture or by feeding it a low-dust diet such as silage or pelleted feed, airway function improves within a few days (Vandenput et al. 1998; Jackson et al. 2000).

Radiolabelling of inflammatory cells has shown that when an RAO-susceptible horse is exposed to hay dust, neutrophils invade the lungs and airways within 4–6 h. Airway obstruction develops concurrently (Fairbairn et al. 1993b). The most intense inflammatory response (peribronchiolar accumulation of lymphocytes and intraluminal accumulation of neutrophils) occurs at the bronchioles (Derkson et al. 1985b; Kaup et al. 1990a,b; McGorum et al. 1993b), but functional changes also occur in the larger airways (Robinson et al. 1999). Bronchoalveolar lavage demonstrates that the principal inflammatory cell in the airway lumen is the neutrophil (Derkson et al. 1985b; McGorum et al. 1993b). Concurrent with the onset of inflammation and airway obstruction, non-specific airway hyperresponsiveness develops (Derkson et al. 1985a; Klein and Deegen 1986). Non-specific airway hyperresponsiveness is an exaggerated bronchospasm occurring in response to a wide variety of stimuli such as inflammatory mediators, neurotransmitters or changes in osmolarity. This airway hyperresponsiveness makes the horse particularly prone to bronchospasm during attacks of airway inflammation. Even brief exposures of susceptible horses to hay dust can initiate airway hyperresponsiveness that can persist for days (Fairbairn et al. 1993a). Administration of bronchodilators to horses with acute attacks of heaves demonstrates that obstruction is due primarily to bronchospasm (Murphy et al. 1980; Robinson et al. 1993; Derksen et al. 1996). However, following maximal bronchodilation, some airway obstruction persists, probably due to accumulation of mucus and exudates, and remodelling of the airway wall. Recent investigations by our group have demonstrated persistent increases in measures of intraluminal mucus even after horses have been at pasture for several weeks. Twenty-four hours after such horses are stabled, the mucus becomes extremely...
viscoelastic and less readily cleared, contributing to mucus accumulation (Gerber et al. 2000).

Bronchospasm in horses with heaves results from facilitation of parasympathetically mediated smooth muscle contraction (Robinson et al. 1993; Duvivier et al. 1997) by inflammatory mediators and decreased inhibition of contraction by inhibitory prostanoids and the iNANC nervous system (Yu et al. 1994). In vitro experiments have demonstrated that activation of neutrophils has no effect on the response of smooth muscle to cholinergic stimulation, but mast cell-derived mediators such as histamine, serotonin and leucotriene D4 greatly facilitate smooth muscle contraction and could be responsible for the cholinergically mediated bronchospasm of heaves (Olszewski et al. 1999).

Measurements of inflammatory mediators in blood and BALF have demonstrated increases in histamine, serotonin, some prostanoids, thromboxane, isoprostanes and 15-HETE (Gray et al. 1989, 1992; Watson et al. 1992; McGregor et al. 1993a; Kirschvink et al. 1999). However, use of specific blockers has not identified a unique role for any mediator in RAO. This is very similar to the situation in asthma and other inflammatory obstructive airway diseases. The importance of inflammation as a cause of airway obstruction is demonstrated by the fact that therapy with corticosteroids, especially dexamethasone, reverses many of the functional consequences of RAO (Lapointe et al. 1993; Rush et al. 1998).

Even though breeding of affected and unaffected horses has shown that there is a genetic predisposition to chronic airway disease (Marti et al. 1991), it is unlikely that heaves is a result of over-expression of a single trait. Elevated levels of IgE in bronchoalveolar lavage fluid support the involvement of a type-1 hypersensitivity reaction (Halliwell et al. 1993), probably a local allergic response to thermophilic molds. However, the immediate (within 15 min) airway obstruction that usually accompanies the release of mast cell-derived mediators during a type-1 reaction is never observed in horses. Attempts to define the predominant T-cell population in blood and bronchoalveolar lavage fluid have identified differences in the total number and ratio of CD4+ and CD8+ T-cells (McGorum et al. 1993a; Watson et al. 1997), but inconsistent findings prevent definitive conclusions at this time.

There are 2 possible neutrophil chemotactic factors involved in heaves: leucotriene B4 and IL-8. The horse lung produces larger amounts of leucotriene B4 than of cysteinyl leucotrienes and leucotriene B4 is chemotactic for neutrophils in horses (Marr et al. 1998; A. Lindberg, unpublished data). However, elevated levels of leucotriene B4 have not been detected in BALF. Furthermore, unpublished data from experiments that blocked leucotriene synthesis and leucotriene receptors suggest that leucotriene B4 is not important in the pathogenesis of heaves. Elevated levels of IL-8 are associated with acute exacerbations of heaves (Franchini et al. 2000) suggesting that IL-8 is a chemotactic factor for neutrophils. Increased expression of NF-κB in bronchial epithelial cells and granulocytes parallels airway obstruction (Bureau et al. 2000b). NF-κB is probably activating genes encoded for IL-8, ICAM-1 and other pro-inflammatory cytokines. Persistent expression of NF-κB by IκB may be due to an imbalance between high levels of IL-1β- and TNFα-mediated IκB degradation and low levels of IκB synthesis (Bureau et al. 2000a). As granulocytes undergo apoptosis, secretion of IL-1β- and TNFα decreases, IκB degradation ceases and newly synthesised IκB binds and inactive NF-κB, thus turning off inflammation.

IAD (Moore et al. 1995) affects 25–30% of horses in training in the USA (Sweeney et al. 1992). In the UK, racehorses spend about 30% of that time in training with IAD and each bout can last for several weeks (Burrell et al. 1996). Epidemiological investigations have implicated streptococcal infections of the larger airways in about 50% of horses with IAD (Wood et al. 1993; Burrell et al. 1996; Chapman et al. 2000) and equine rhinovirus in another small percentage. In some horses, no infectious cause can be identified and environmental factors are thought to be the aetiological agents. It is highly likely that in young horses, as in young people, there can be numerous causes of airway inflammation: bacterial, viral, allergic and environmental. Neutrophils are the predominant inflammatory cell, but occasional horses have increased numbers of eosinophils or mast cells (Viel 1997; Hare and Viel 1998). The relevance of these different cell types to the aetiology and treatment of IAD is currently unknown. At present, there is no evidence to connect IAD with RAO in the old horse and there is no way to identify a young horse that will develop RAO later in life (Robinson et al. 2001).

Further advances in understanding the pathogenesis of both RAO and IAD require in-
depth investigation of the inflammatory process in horse airways and its immunological basis. The goal should be early identification of markers that will predict which horses will develop heaves later in life. Once such horses are identified, they could be managed to prevent exposure to dusts and the development of severe lung disease.

REFERENCES


Immunology in 2001


Horses of all ages can be afflicted with non-infectious airway disease (NIAD) that manifests with varying clinical, physiological and pathological findings. The common feature is obstruction of the airways due to accumulation of secretions, thickening of the airway wall and, in advanced cases, loss of radial traction at the level of the terminal airways. Longitudinal epidemiological studies have not yet been conducted to assess the relationship between disease and age, and this abstract outlines the 3 major phenotypes associated with NIAD: horses with heaves, poorly performing young horses and foals.

In general, the clinical signs observed in NIAD vary with the severity of the condition and age of the animal. Intermittent cough at rest or the beginning of exercise, early signs of fatigue during exercise and a persistently elevated respiratory rate post exercise are often seen in foals and athletic horses. The detection of crackles and wheezes on auscultation and significantly expanded lung field on percussion are additional features. Nasal discharge, nasal flaring and abdominal expiratory effort are mostly observed in older horses, or those with heaves, but can occur in foals with severe airway inflammation. A diagnostic aid to compliment clinical examination is endoscopy of the airway. This allows clinicians not only to score the amount of mucopus in the large airways, but also to recognise mucosal oedema and lumen closure due to bronchoconstriction. To assess the severity of disease quantitatively, from mild to severe, indices for clinical and endoscopic scoring represent important tools for direct correlation between endoscopic findings and other pulmonary physiological and pathological parameters (Hare et al. 1994; Tesarowski et al. 1996).

There is consistent evidence that pathological lesions in NIAD are localised in the non-cartilagenous small airways or bronchioles (Viel 1985; Winder 1988). The characteristic histological changes consist of epithelial cell hyperplasia and metaplasia, peribronchiolar inflammatory cell infiltrate, lymphoid cell hyperplasia, smooth muscle hypertrophy and airway lumen occlusion by mucopus casts. Fibrosis and alveolar thickening may be observed in advanced cases of heaves. It is of interest that, in a study where histological parameters were assessed semi-quantitatively between normal and mild-to-severe cases of NIAD, 4 significantly different subgroups could be identified (Table 1). The data indicated that degree of lesion severity was closely associated with age of the animal. Further, horses affected with NIAD had significant pathological small airway changes prior to detection of abnormal pulmonary function parameters such as altered transpulmonary pressure and dynamic compliance.

**TABLE 1: Pathology and lung function in horses with NIAD**

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>1 (11)</th>
<th>2 (40)</th>
<th>3 (7)</th>
<th>4 (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>5±0.8</td>
<td>6±1.8</td>
<td>8±1.6</td>
<td>12±1.5</td>
</tr>
<tr>
<td>Histological score</td>
<td>39±3</td>
<td>90±4</td>
<td>157±5</td>
<td>222±5</td>
</tr>
<tr>
<td>Dppl (cm H2O)</td>
<td>5±1</td>
<td>6±1</td>
<td>7±3</td>
<td>15±7</td>
</tr>
<tr>
<td>Cdyn (L/cm H2O)</td>
<td>2.1±0.2</td>
<td>2.1±0.4</td>
<td>1.8±0.4</td>
<td>1±0.2</td>
</tr>
</tbody>
</table>

From: (Viel 1985)
Airway obstruction resulting from severe bronchiolitis is compounded further by accumulation of excessive inflammatory cells and mucus within the airway lumen. In well-characterised cases of heaves in horses >10 years old, a significantly elevated mixed inflammatory cell population is retrieved from bronchoalveolar lavage (BAL). Neutrophils frequently account for over one third of the total inflammatory cell differential in such cases, and play a pivotal role in the pronounced airway hyperreactivity. The massive influx and persistence of neutrophils within the lumen of the airways is reflective of ‘heavey’ horses in a state of disease exacerbation. Exacerbation can be documented further by a 35% drop in dynamic compliance following aerosol exposure to low levels of histamine (<8 mg/ml).

In contrast to heaves in older horses, athletic horses aged 2–5 years show a remarkable degree of airway hyperreactivity to inhaled doses of histamine as low as 5 mg/ml (Hare et al. 1998) and 6 mg/ml (Hoffman et al. 1998). BAL fluid from such horses demonstrates the presence of increased eosinophils and mast cells, as well as a slight increase in the number of neutrophils and lymphocytes.

Little emphasis has been placed on the concept that frequently encountered airway inflammatory disorders may reflect persistent inflammation rather than ongoing bacterial or viral infection. This is best exemplified by discussing respiratory disease in foals. In general, clinicians assume that purulent exudate at the nares or seen on endoscopic examination of a foal directly implies an active bacterial infection (eg Streptococcus zooepidemicus). However, these exudates may actually reflect ongoing, sterile airway inflammation despite resolution of the underlying infectious process. Cytologically, airway inflammation is indicated by large numbers of neutrophils identified in BAL fluid (Hoffman et al. 1993). In a report by Lakritz et al. (1993) on 1- to 7-month-old foals with acute bronchointerstitial pneumonia and/or respiratory distress, 9 of 10 treated aggressively with corticosteroids survived, supporting the theory that airway inflammation plays an important role in the pathophysiology of this condition rather than simply the persistence of an infectious agent. Further, pathological findings in foals submitted for necropsy from the same study indicated the presence of subclinical chronic inflammatory small airway disease. It is unknown if these airway changes are transient or permanent in nature, but they typically respond well to corticosteroid therapy. Physiologically, there is supporting evidence that affected foals of the same age group as the one described by Lakritz have changes in their pulmonary function parameters, including airway hyperresponsiveness to aerosolised histamine and the presence of reduced arterial oxygen tension (hypoxemia) (Hoffman et al. 1999).

In summary, although all 3 phenotypes of NIAD share some common abnormalities in their clinical, physiological and pathological findings, our understanding of NIAD in general is hampered by the lack of a cohesive, integrated approach in studying these phenotypes.

**REFERENCES**


Neutrophils have a critical role as a first line of defence against host insult from pathogens and inhaled agents and, consequently, individuals with neutrophil dysfunction may develop life-threatening pneumonia. Because neutrophils are almost absent from the normal lung, to facilitate this protective role, peripheral blood neutrophils must be primed and recruited to the lung by chemoattractants and chemokines such as interleukin-8 (IL-8), macrophage inflammatory protein-2 (MIP-2), tumour necrosis factor-α (TNF-α), leucotriene B4 (LTB4) and bacterial products (Sibille and Reynolds 1990). Transmigration of neutrophils from the pulmonary vasculature through the basement membrane into the airways is facilitated by proteinases including elastase and metalloproteinases (MMPs). Once recruited, neutrophils may be activated by inflammatory and bacterial agents, resulting in a respiratory burst (a marked increase in production of reactive oxygen species) and degranulation, with release of a wide array of antimicrobial agents including proteases and cationic proteins, in addition to inflammatory mediators and cytokines. Unfortunately neutrophils contain more than 30 potentially histotoxic agents which may stimulate further recruitment, activation and degranulation of inflammatory cells, promote mucus hypersecretion, increase pulmonary vascular permeability and ultimately cause host tissue damage (Haslett et al. 1989). Neutrophil mediated host damage is considered to be important in the pathogenesis of human acute respiratory distress syndrome and chronic obstructive pulmonary disease. Consequently, the function of neutrophils must be regulated tightly, and the potentially detrimental effects of neutrophil contents are limited by several control mechanisms including inactivation of neutrophil products by antiproteinases and antioxidants, and the removal of intact apoptotic neutrophils by macrophages and by mucociliary clearance.

Equine heaves (previously termed chronic obstructive pulmonary disease) is characterised by a neutrophilic endobronchitis, with bronchoalveolar lavage fluid (BALF) neutrophil numbers being increased by up to 50-fold. Airway neutrophil recruitment can be induced in heaves horses by hay/straw exposure, and by inhalation of aqueous mould extracts and endotoxin. There is evidence to support involvement of IL-8, MIP-2, LTB4 and platelet activating factor (PAF) in equine pulmonary neutrophil recruitment (Franchini et al. 1998; Marr et al. 1998; Brazil 1999). In people with grain dust-induced occupational asthma, IL-8 has been implicated as a prime candidate for induction of neutrophil recruitment (Park et al. 1998). Increased levels of active MMP-9 in airways of heaves horses is consistent with the crucial role of MMPs in the transmigration of inflammatory cells through the basement membrane into the airways (Raulo et al. 2001). Indeed, consistent with this role, the magnitude of airway neutrophilia was closely correlated with the BALF concentrations of pro-MMP-9 and active MMP-9, but not with the BALF MMP-2 concentration (Nevalainen et al. 2001). Activation of MMP-9, which is a prerequisite to its biological effect, may be mediated by either oxidants or proteinases, and there is evidence for both reactive oxygen species (ROS)- and protease-mediated MMP-9 activation in heaves (Nevalainen et al. 2001).

Although neutrophils are the predominant inflammatory cell in the airways of heaves affected horses, their presence per se does not necessarily imply involvement in airway obstruction. Indeed Fairbairn et al. (1993) demonstrated that in some
heaves horses, airway dysfunction preceded detectable pulmonary neutrophil sequestration. Furthermore, although inhaled endotoxin (Pirie et al. 2001) and Faenia rectivirgula (Derkson et al. 1988) induced a marked neutrophilia, this was not always associated with concomitant airway dysfunction.

Peripheral blood neutrophils are primed following antigen challenge in heaves horses, as indicated by increased basal and agonist stimulated superoxide production (Marr et al. 1997; Brazil 1999). Furthermore, the neutrophils within the air spaces are primed and activated, as evidenced by their increased respiratory burst in response to the bacterial tripeptide fMLP, and by the increased BALF concentrations of neutrophil degranulation products including neutrophil elastase and MMP-9 (Brazil 1999; Raulo et al. 2001; Nevalainen et al. 2001). In addition, the finding that heaves is associated with oxidative stress (Art et al. 1999), and that the degree of oxidative stress is correlated with the airway neutrophil count, is consistent with increased production of ROS by neutrophils, although these may also be derived from other activated leucocytes such as macrophages.

Although the neutrophil elastase present in BALF from heaves horses is inactivated, probably due to inhibition by proteinase inhibitors such as alpha-1-proteinase inhibitor (API), it may contribute to histotoxic damage by acting locally within the immediate pericellular microenvironment prior to interaction with inhibitors (Brazil 1999). In contrast, as much of the MMP-9 in BALF is present in the active form, it may have a more global effect leading to damage and remodelling of the airway epithelium, basement membrane and pulmonary interstitium. Horses with heaves may have minor degrees of pulmonary emphysema, with the severity of histological changes relating to the degree and extent of clinical disease (Kaup et al. 1990). The extent of airway remodelling is considerably less that that which characterises human chronic obstructive pulmonary disease. This inter-species difference may, in part, reflect improved efficacy of the equine antiproteinase screen. In this respect, several potentially important interspecies differences in the nature and function of one of the major proteinase inhibitors, namely API, have been identified. In contrast to human API, which is encoded by a single gene, equine API is encoded by 4 alleles producing 4 proteins termed Spi 1-4. To date 22 haplotypes have been identified, but heaves does not appear to be associated with any particular haplotype. Most of the API in equine BALF is derived from plasma API. API levels in normal equine BALF are 2–3-fold higher than those in man, and are increased further in horses with heaves (Milne et al. 1994). As equine Spi 3, which comprises a significant proportion of the equine serpin shield, is resistant to the oxidative inactivation that affects human API, the horse may have adequate antiprotease activity in local environments that are rich in ROS. Furthermore, and again in contrast to the situation in man, the equine API complex is not chemoattractant for equine neutrophils, and hence is unlikely to lead to further pulmonary recruitment of neutrophils (Scudamore et al. 1993). The effect of neutrophil products on airway smooth muscle function is as yet unclear, and is likely to be multifactorial (Olszewski et al. 1995; Art et al. 1999). Although neutrophil derived products, including elastase, contribute to goblet cell degranulation in antigen challenged guinea pigs (Agusti et al. 1998), it is not yet known whether they contribute to the mucus hyper-secretion in heaves.

In the resolution phase of heaves there is an increase in the proportion of neutrophils undergoing apoptosis (programmed cell death) in the airspaces. These neutrophils are removed subsequently via phagocytosis by airway macrophages (Brazil 1999), or cleared via the mucociliary escalator. As apoptosis results in down-regulation of a number of neutrophil functions including chemotaxis, phagocytosis and granule release, this process serves to limit further host tissue damage and promote resolution of inflammation (Whyte et al. 1993). However, although apoptosis undoubtedly contributes to clearance of neutrophils from the lungs of heaves horses, the constitutive rate of neutrophil apoptosis appears to be reduced during an exacerbation of heaves (Brazil 1999). This inhibition of apoptosis probably results from the inhibitory effects of pro-inflammatory agents including cytokines, which may enhance neutrophil longevity via upregulation of the nuclear factor-kappa B transcription factor (Bureau et al. 2000).

REFERENCES


Recurrent airway obstruction (RAO) of horses, also known as ‘heaves’, and chronic obstructive pulmonary disease (COPD), is a condition affecting the equine lung in Europe and the United States. Two forms of this disease have been identified based on seasonal occurrences, RAO and summer pasture-associated obstructive pulmonary disease (SPAOPD), although clinical signs and cellular composition of bronchoalveolar lavage fluid suggest a common aetiopathogenesis. Clinical signs of heaves result from small airway inflammation leading to terminal airway obstruction, primarily due to bronchospasm, mucus plugs and airway remodelling affecting the bronchiolar walls. The finding that exacerbation of heaves could be provoked by exposure to dusty hay led researchers to postulate that heaves is an allergic reaction to inhaled moulds and fungi. However, to date, the immunological events leading to airway inflammation in heaves remain ill defined.

Recent findings using molecular tools and murine animal models have highlighted the complex interactions involved in the modulation of allergic lung disease. It is now clear that most cells present within the lung tissue, including epithelial cells, myocytes and nerve endings in addition to traditional inflammatory cells, contribute to this modulation. Among these, T cells (CD4+ cells, more specifically of Th2 cells) play a central role in allergic airway inflammation. Cytokines produced by Th2 cells, such as interleukin (IL)-4, IL-5 and IL-13 have been implicated in allergic inflammation. Th1 cells are important for cell-mediated immunity by their production of INF-γ and other cytokines. Although a polarisation of T helper cells into predominantly Th1 and Th2 subsets has not been investigated thoroughly in horses, in vitro culture of equine CD4+ T cells following chronic allergen stimulation has induced a Th2 cytokine profile characterised by an increased expression of IL-4 mRNA and a decreased expression of IL-2 mRNA (Aggarwal et al. 1999).

Lower airway inflammation, reversible airway obstruction and bronchial hyperresponsiveness are characteristic of heaves in horses and human asthma. As asthma is associated with a Th2-type cytokine expression in bronchoalveolar (BAL) cells (Robinson et al. 1992) and because the 2 diseases share many similarities, we postulated that heaves also has a Th2 type cytokine expression in BAL cells. Using in situ hybridisation, we found that BAL cells from horses with heaves chronically exposed (months) to dusty hay have increased expression of IL-4 and IL-5 mRNA and decreased expression of INF-γ compared to controls, which is consistent with a Th2 type cytokine response (Lavoie et al. 2000). IL-4 promotes the development and the growth of Th2 cell phenotype and is essential for the induction of B-cell isotype switching to IgE antibody production (Lasky and Brody 1997). The increase in IL-4 mRNA expression in heaves is consistent with the elevated levels of antigen specific IgE in serum and BAL of horses with heaves (Halliwell et al. 1993; Schmallenbach et al. 1998; Eder et al. 2000). The results of IL-5 mRNA expression in the present study are less clear. An increased expression of IL-5 mRNA is usually associated with tissue eosinophil migration, which is not a common feature in heaves. The presence of IL-5 within the airway lumen may not be sufficient by itself to induce a local eosinophilia, as suggested by the results of a study in a murine model, showing that circulating but not local IL-5 was associated with pulmonary eosinophilia (Wang et al. 1998). It is also possible that IL-5
mRNA expression may not have been associated with protein production in the present study, as it occurred in human bronchitis (Jeffery et al. 1999). However, when using RT-PCR on BAL cells of horses with heaves compared to controls, no consistent findings on cytokine mRNA expression were found in 3 preliminary reports (Lavoie et al. 1999; Ainsworth et al. 2000; Giguere et al. 2000) and IL-5 mRNA was not detected in 2 of them (Lavoie et al. 1999; Giguere et al. 2000). Quantitative PCR analysis of horses affected with SPAOPD demonstrated elevated levels of mRNA for IL-4 and IL-13 during that time of the year (summer) when clinical symptoms were present (Horohov 2000). Control animals exhibited a bias towards Th1 cytokine (interferon-γ) production in their lungs. We also failed to detect elevated levels of mRNA for IL-5 in the lungs of affected horses. While half of the affected animals returned to a Th1 bias in their mRNA profile in the winter, the others exhibited elevated signs of IL-4 and IL-13 mRNA, suggesting a more chronic condition in these animals. The discrepancies in results between studies clearly indicate that more work will be required before a definite conclusion can be reached concerning the contribution of Th1/Th2 type cytokines to heaves and SPAOPD.

A number of additional cytokines and chemokines likely to be implicated in the modulation of airway inflammation in heaves are being investigated. Of particular interest, the role of cytokines secreted by epithelial cells, neutrophils and macrophages may play a crucial role in airway remodelling and secretions involved in heaves. It has been shown that BAL fluids of ‘heavey’ horses have increased chemotactic activity for neutrophils (Franchini et al. 1998, 2000) and that IL-8 mRNA expression (Lavoie et al. 1999) and IL-8 concentration (Franchini et al. 2000) are also increased.

**References**


IgG SUBTYPES AND CLUES TO THE IMMUNOPATHOLOGY OF RECURRENT AIRWAY OBSTRUCTION (HEAVES)

D. M. Ainsworth, J. A. Appleton*, D. F. Antczak*, M. A. Santiago and G. A. Aviza

**Department of Clinical Sciences; *Department of Microbiology & Immunology and James A Baker Institute for Animal Health; Cornell University, Ithaca, New York 14853, USA**

Heaves is an inflammatory condition of the small airways of middle-aged and older horses stabled on straw and fed hay. The ailment is life-long, it is debilitating, and it has a negative effect on the athletic usefulness of susceptible horses (Aviza et al. 2001). The pathogenesis of heaves currently remains unknown but it is widely believed to represent an immune-mediated disease triggered by exposure to environmental moulds or dust found in hay and bedding. The moulds implicated most commonly are *Aspergillus fumigatus*, *Faenia rectivirgula* and *Thermoactinomyces vulgaris*.

Understanding the nature of this immune-mediated disease of heavey horses becomes important for therapeutic and prophylactic interventions. This is especially applicable to North American show horses for which environmental management changes are not feasible and for which the administration of glucocorticoid therapy is not permissible.

Studies demonstrating elevated bronchoalveolar lavage fluid (BALF) and serum IgE levels against *A. fumigatus* and *F. rectivirgula* in affected horses provide data suggesting that heaves is a type 2 (T helper cell–2) immune response (Eder et al. 2000; Halliwell et al. 1993; Schmallenbach et al. 1998). In contrast, studies demonstrating: 1) a lack of correlation between antigen-specific IgE levels and expression of the disease (Dixon et al. 1996; Eder et al. 2000); 2) a lack of early phase BALF histamine release in naturally challenged heavey horses (McGorum et al. 1993); and 3) a lack of development of pulmonary eosinophilia in heavey horses (Derksen et al. 1985), provide data supporting the hypothesis that heaves is a Th-1 immune response.

One approach to investigating the type of immune responses existing within the respiratory tract of heavey horses would be to measure the pulmonary and systemic antibody responses to a nebulised novel foreign antigen. Currently, measurement of equine BALF and serum IgE is confounded by the lack of readily available reagents. However, in most species, type 2 cytokines promote B lymphocyte switching not only to IgE, but also to selected IgG sub-isotypes (Coffman et al. 1993; Snapper et al. 1997). In the horse, the IgG sub-isotype most likely to be associated with a Th-2 response is IgG(T) whereas the IgG sub-isotypes most commonly associated with a Th-1 response are IgGa and IgGb (Nelson et al. 1998). Thus, using the context of a natural challenge (NC) environment (dusty hay, indoor stabling on straw), we sought to determine if the chronic inflammatory events existing within the respiratory system of heavey horses would bias IgG subtype formation in response to a nebulised foreign antigen, keyhole limpet hemocyanin (KLH). The aim was to establish whether, in healthy and affected horses, the KLH sub-isotypes formed reflect a Th-1 or Th-2 immune response.

Pulmonary function testing and BALF neutrophil populations were used to assign horses to control or heaves-affected groups. There was no difference in the median age of the 2 groups. Baseline sera and BALF fluid (Sample 1) were obtained on Day 14 of natural challenge environment. Horses were then nebulised 4 times (at 3 day intervals) with a 0.1% solution of KLH in saline. Sera and BALF were collected 18 and 32 days later (Samples 2 and 3). KLH-specific antibody levels were measured using an ELISA developed and adapted using isotype-specific mouse monoclonal antibodies. The 4 sub-isotypes quantified were IgGa, IgGb, IgG(T) and total IgG. Differences between the 2 groups were detected using non-parametric analysis with P<0.05 selected for significance.
We found that anti-KLH IgG levels were significantly greater in BALF and sera from control horses compared to diseased horses. Differences in sub-isotypic responses between the 2 groups were also evident. BALF anti-KLH IgGb levels in control horses exceeded those of diseased horses, but BALF anti-KLH IgG(T) levels in diseased horses were not significantly increased relative to controls.

In contrast to the prevailing notion that horses with heaves have hyperreactive immune responses, our data suggest that diseased horses mount a weak IgG response compared to healthy horses. Differences in IgG levels between the 2 groups might be attributed to down-regulation of type 1 cytokines. However, as the anti-KLH IgG(T) levels of the heavey horses failed to exceed those of the controls, it seems unlikely that heaves reflects a Th-2 response.

Differences in the antibody responses between the 2 groups of horses may also be attributed to: 1) the large numbers of neutrophils in the lungs of diseased horses that would bind available IgGa and IgGb via Fc receptors (McGuire et al. 1973); and/or 2) protease-induced destruction of the antigen in the lungs of heavyy horses (Koivunen et al. 1996). Based upon the results of our study, we were unable to conclude whether the pulmonary immune environment of heavyy horses reflects a Th-1 or Th-2 process. (This work was supported by the Harry M. Zweig Memorial Fund for Equine Research).

REFERENCES


The purpose of the study was to assess the effects of airborne dust and endotoxin exposures on lung function and airway cytology in horses maintained in environmentally controlled rooms. Four horses were housed for 2-week periods in each of 3 environments: low dust (LD, pelleted feed and wood shavings), medium dust (MD, pelleted feed and straw) and high dust (HD, mouldy hay and straw). Lung mechanics, airway responsiveness and bronchoalveolar lavage fluid cytology were assessed at the end of each exposure period. Total and respirable dust and endotoxin levels were monitored in the horse’s breathing zone for each environment.

Dust exposure levels were significantly greater in HD than in MD or LD environments for both total dust (6.84 ± 1.93, 2.12 ± 0.18, 1.46 ± 0.69 mg/m³, respectively) and respirable dust (1.85 ± 1.11, < 0.003, 0.182 ± 0.244 mg/m³, respectively). Endotoxin exposure levels in respirable dust were significantly higher in the HD than in MD and LD environments (9.21 ± 2.07, 4.88 ± 3.39, 1.50 ± 1.86 ng/m³, respectively). No significant differences in lung function were detected in horses after exposure to HD, MD and LD environments. There was a trend for an increase in airway responsiveness to histamine challenge when horses were placed in the HD environment. The percentage of neutrophils in the bronchoalveolar lavage fluid was significantly higher in HD than in MD and LD environments (15.8 ± 4.9, 3.0 ± 1.83, 2.8 ± 2.1 %, respectively).

We conclude that horses placed for 2 weeks in HD environment exhibited significant airway inflammation as a result of high exposure levels to respirable dust and endotoxin. However, the effect of dust and endotoxin exposure levels on lung function was marginal.
More than 100 years ago, it was discovered that heat stable extracts of Gram-negative enteric bacteria were highly toxic. Based on the assumption that these toxins were released from the interior of the bacteria upon their death, the term ‘endotoxin’ was coined. The results of subsequent biochemical studies, however, determined that these endotoxins do not arise from the interior of the bacteria, but are lipopolysaccharide (LPS) components of the outer membrane of enteric bacteria. Furthermore, it was determined that LPS is comprised of 3 structural units: an outer polysaccharide component, a core oligosaccharide region and the innermost portion, lipid A, which affords the molecule its pro-inflammatory activities.

Although the gastrointestinal tract has a very efficient mucosal barrier to restrict LPS to the intestinal lumen, gastrointestinal diseases which compromise this mucosal barrier often result in movement of LPS into the bloodstream. Using the highly sensitive limulus amoebocyte lysate assay to detect LPS, it has been determined that 35–45% of horses presented to veterinary colleges with colic are endotoxaemic, that most endotoxaemic horses have intestinal strangulation obstruction or severe inflammatory intestinal diseases and that prognosis for survival is inversely correlated with the presence of LPS in circulation. The situation is equally serious in neonatal foals that develop a nidus of bacterial infection, such as an infected umbilical remnant. In a recent clinical study of 34 neonatal foals with presumed septicaemia, LPS was detected in the plasma of 50% of septic foals sampled and derangements in hemostasis and fibrinolysis were correlated with the presence of endotoxin in circulation. Thus, endotoxaemia is associated with the primary causes of death in horses of all ages.

The clinical evidence cited above has led a variety of workers to investigate the effects of LPS in horses, either by studying the effects of the toxins in vivo or in vitro. The results of numerous studies performed in a variety of species, including the horse, indicate that endotoxic shock is initiated when the host’s mononuclear phagocytes interact with LPS and synthesise pro-inflammatory mediators. Under experimental conditions, serum concentrations of the cytokine tumour necrosis factor (TNF) increase to peak values within 2 h after onset of endotoxaemia; and serum TNF activity has been associated with the onset of signs of abdominal pain, fever and leucopenia. In clinical studies of horses with colic and foals with septicaemia, increased serum concentrations of TNF activity have been identified and associated with mortality rate.

Similarly, numerous studies have documented the role of lipid-derived mediators, principally cyclo-oxygenase products of arachidonic acid metabolism, in endotoxaemia in horses. The results of these studies suggest that many of the early haemodynamic and behavioural effects of endotoxaemia are due to thromboxane A₂, and prostaglandins E₂, F₂α and I₂. As a logical extension of these studies, beneficial effects of potent non-steroidal anti-inflammatory drugs have been reported in horses and ponies with endotoxaemia in experimental studies. These results form the basis for the use of these drugs in clinically ill horses and foals with conditions characterised by endotoxaemia.

Alterations in coagulation and fibrinolytic factors in endotoxaemia have been identified, either by in vitro studies with isolated monocytes or peritoneal macrophages, or by measuring these factors in plasma from horses and foals with naturally occurring diseases characterised by
endotoxaemia. In clinical studies of horses with colic, significant decreases in plasma antithrombin-III activity, protein C and plasminogen activity were identified. Expression of tissue factor by monocytes isolated from horses with colic was significantly greater than expression by monocytes from healthy control horses. Similarly, the results of a recent study of neonatal foals with presumed septicaemia indicated that coagulation times tend to be prolonged in endotoxaemic foals, compared to values for age-matched healthy foals and septic foals in which endotoxin is not present in the plasma. These findings indicate the presence of a hypercoagulable state in endotoxaemic horses with colic and foals with septicaemia.

Although there is convincing evidence that endotoxaemia occurs in horses and foals and causes the synthesis of pro-inflammatory mediators that lead to many of the complications encountered in clinical practice, the molecular mechanisms by which endotoxin exerts its effects in horses are largely unknown. This is not the case for other species, for which it is known that LPS in the circulation binds rapidly to a unique plasma protein named lipopolysaccharide binding protein (LPS-binding protein). LPS-binding protein, which is synthesised by hepatocytes as part of the acute phase response of inflammation, has a strong affinity for the lipid A portion of endotoxin, as well as for CD14, a glycosylphosphatidylinositol-anchored receptor on the surface of mononuclear phagocytes. When LPS and LPS-binding protein interact with CD14, these cells become activated and produce a variety of pro-inflammatory mediators. Alternatively, LPS and LPS-binding protein may interact with a soluble form of CD14 in circulation and then activate cells lacking membrane-bound CD14.

Because CD14 lacks a transmembrane component, the endotoxin signal must be transduced through something other than CD14. The results of more than 250 published studies since 1998, when the observation was first made, indicate that the endotoxin signal is transmitted to the interior of the cell via the Toll-like 4 transmembrane receptor. With these new findings in mind, potential new therapeutic interventions that warrant study include mechanisms to interfere with the binding of LPS to LPS-binding protein, or CD14 or to prevent the intracellular responses initiated by activation of Toll-like receptor 4.
The purpose of the studies reported here was to address the hypothesis that an imbalance between endothelium-derived vasodilator (nitric oxide, NO) and vasoconstrictor (endothelin 1, ET-1) substances is the initiating factor in the development of acute laminitis in horses. Although the pathophysiology of this debilitating, excruciatingly painful and potentially career-ending and life threatening disease of the soft tissues of the foot is not completely understood, there is substantial evidence suggesting that local digital haemodynamic alterations play a role in its initiation. Acute laminitis is characterised pathophysiologically by decreased pre-to-post capillary resistance ratio and increased capillary pressure subsequent to increased venoconstriction; increased laminar interstitial pressure and oedema formation; microvascular compression; thrombosis and formation of cellular (platelet-platelet and platelet-neutrophil) aggregates; and decreased digital blood flow and laminar perfusion which ultimately leads to laminar ischaemia, inflammation and necrosis. Our current lack of a complete understanding of acute laminitis prohibits effective therapeutic and preventive measures from being developed and employed in the clinical management of this disease.

Laminitis occurs in adult horses and often occurs secondarily to diseases typically associated with endotoxaemia. Although experimental administration of endotoxin has never been demonstrated to cause laminitis, its administration is associated with appreciable decreases in digital blood flow and laminar perfusion. Additionally, endotoxin may cause endothelial damage and result in decreased NO and increased ET-1 synthesis and release. Endotoxaemia has been associated with alterations in plasma and tissue NO and ET-1 concentrations in other species. NO is synthesised constitutively by endothelial NO synthase and causes vascular smooth muscle relaxation, decreased neutrophil adhesion and decreased platelet aggregation and adhesion. ET-1 is a peptide synthesised by the endothelium and is the most potent vasoconstrictor substance known; it is also a mitogen and stimulates platelet aggregation. Because of the physiological and pathophysiological properties of NO and ET-1, an imbalance between these substances (decreased NO and increased ET-1) could account for initiation and propagation of many of the above mentioned alterations known to occur with acute laminitis.

Our laboratory began investigating this hypothesis using an in vitro study of the effects of ET-1 and non-selective ET antagonists on contractile properties of palmar digital artery and veins collected from normal, non-laminitic horses. In this study, we investigated the hypotheses that ET-1 would cause a marked contraction of arteries and veins, that the response would be greater in veins, and that this contraction could be prevented by pre-treatment with ET antagonists. The results demonstrated that ET-1 causes a slowly developing, but sustained and profound, dose-dependent contraction of arteries and veins, that the response would be greater in veins, and that this contraction could be prevented by pre-treatment with ET antagonists. The results demonstrated that ET-1 causes a slowly developing, but sustained and profound, dose-dependent contraction of arteries and veins. Digital veins were much more sensitive and responded to a magnitude 3.5 times greater than that observed in arteries. Pre-treatment of vessels with either of the antagonists caused a dose-dependent blockade of the contraction, although the B-2 antagonist (PD145065) was more effective than the B-1 antagonist (PD142893) and caused complete blockade.

A preliminary in vitro vessel study in 4 horses with naturally acquired laminitis suggested that digital veins were more sensitive to the effects of ET-1 than in normal horses. The B-2 antagonist
was effective in greatly reducing the contraction in at least some arteries and veins of these horses.

To further evaluate the effects of ET-1 and ET antagonists on the response of the normal digital vasculature of horses, we evaluated these substances in an in vivo study in conscious horses with a surgically implanted ultrasonic digital flow probe around the lateral palmar digital artery. We investigated the hypotheses that ET-1 infusion into the arterial circulation of horses would cause a dose-dependent reduction in digital blood flow and that this could be prevented and/or reversed with administration of the ET antagonist B-2 when infused into the digital arterial circulation as pre-treatment or after ET-1, respectively. This study confirmed that ET-1 infusion into the digital artery caused a dose-dependent reduction in digital arterial blood flow. Additionally, at higher doses, ET-1 caused digital pain that was marked in most horses at the highest concentration (10-6 M). Pretreatment with a 10-5 M concentration of B-2 effectively blocked the ET-induced decrease in digital blood flow. In another experiment in the same horses, a dose-response study of B-2 infusion was performed after digital blood flow was decreased by pre-treatment with ET-1. There was a dose-dependent improvement in blood flow and at the highest B-2 concentration (10-5 M), there was a significant improvement in blood flow.

To assess the effects of ET-1 on the digital microvasculature, we have performed a preliminary study of the digital Starling forces in 3 horses. In this study, we infused ET-1 at a dose comparable to 10-6 M into the digital arterial circulation. ET-1 caused a decrease in digital blood flow due to an increase in vascular resistance; the increased vascular resistance was associated with a 30% increase in post capillary resistance. This suggests venous constriction is an important component of the increase in digital vascular resistance and decreased digital blood flow observed with ET-1 infusion. Capillary pressure increased from 36 to 52 mm Hg.

ET-1 immunohistochemical staining of formalin-fixed, paraffin-embedded laminar tissue specimens from laminitic and normal horses demonstrated intense staining of the arteriolar and venous endothelium and the laminar epithelium and stroma of the sensitive laminae in laminitic horses, but not in normal horses. This information corresponds with previous reports of increased ET-1 expression in the laminar tissue of horses with naturally acquired chronic laminitis and in horses with experimentally induced acute laminitis.

Collectively, the findings from these studies suggest that a local increase in digital vascular and laminar ET-1 could account for many of the alterations that occur in acute laminitis. Therefore, the role of ET-1 in the initiation and propagation of acute laminitis is currently being investigated both in the carbohydrate overload and black walnut extract models of experimentally induced acute laminitis. These studies currently focus on measuring digital venous plasma ET-1 concentrations; measuring systemic and digital haemodynamics; monitoring clinical signs; evaluating ET-1 immunohistochemical staining of specimens of laminar tissue and digital arteries and veins; and measuring digital Starling forces in horses given either B-2 or a saline control solution after induction of laminitis.

The information gained from these studies provide important information on the potential role of ET-1 in the pathophysiology of acute laminitis, and the potential therapeutic implications for ET antagonists in horses with naturally acquired disease.
Acute intestinal crises in the horse are life-threatening disorders with intestinal ischaemia or inflammatory bowel disease being the most common. In these disorders, the absorption of endotoxin from enteric bacteria causes the adherence of leucocytes to vascular walls, release of inflammatory mediators, loss of fluid from the vasculature, thrombosis of vessels and necrosis of the intestinal mucosa. For these reasons, fatality rates for horses with acute abdominal crises often approaches 70%.

Adhesion molecules that coordinate signalling pathways and production of cytokines are central to the recruitment and activation of leucocytes at sites of inflammation. Recently, several *in vivo* and *in vitro* experiments have studied microvascular injury during intestinal ischaemia/reperfusion injury and endotoxin-mediated acute lung injury in the horse and pig (Darien *et al.* 1991, 1993, 1995, 1996, 1997, 1998; Triplett *et al.* 1996; McAnulty *et al.* 1997; Kruse-Elliott *et al.* 1998). One mechanism central to mediating tissue necrosis is leucocyte adhesion, transendothelial cell migration and inflammatory mediator biosynthesis (Figs 1 and 2). To this end, we have studied extensively the biosynthesis of 2 chemokines central to leucocyte recruitment, interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) in human monocytes following adhesion to P-selectin via its natural ligand, P-selectin glycoprotein ligand-1 (PSGL-1).

Despite recent progress in understanding its molecular aspects and development of novel...
therapeutics, there remains high mortality in horses with severe abdominal crises such as ascending colon volvulus (ACV), salmonella, and Potomac horse fever. Central to the systemic inflammatory response to these disorders, the pathogenesis of intestinal injury and dysfunction is the activation of leucocytes and vascular cells (Darien et al. 1991, 1993, 1995; Chang 1992; Welbourn and Young 1992). The interaction of activated leucocytes with endothelial cell adhesion molecules plays a major role in the release of soluble mediators, including interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1), which amplify the systemic inflammatory response (Lorant et al. 1993; Granger and Kubes 1994; Lukacs et al. 1995). As such, leucocytes which are recruited for host defence in the early stages of acute inflammatory processes, such as ischaemia, reperfusion and septic shock, are also implicated in the vascular injury and tissue damage of the inflammatory processes of ischaemia/reperfusion injury, multiple organ failure and death (Weyrich et al. 1995; Diacovo et al. 1996).

P-selectin is a member of a family of transmembrane adhesion molecules expressed on a variety of cell types which mediates rapid transient interactions between leucocytes and activated platelets and endothelial cells called ‘rolling’. P-selectin is stored in α-granules in platelets and Weibel-Palade bodies in endothelial cells and expressed on the surface of these cells in response to various stimuli, such as endotoxin (LPS), thrombin and tumour necrosis factor-α (TNF-α). P-selectin binds to leucocytes via glycoproteins expressing sialylated and fucosylated glycans, such as P-selectin glycoprotein ligand-1 (PSGL-1). This transient contact is generally believed to be a pre-requisite for firm adhesion and subsequent extravasation of circulating monocytes and neutrophils, which is central to the pathogenesis of vascular injury during endotoxaemia and inflammatory or ischaemic intestinal disorders. While the P-selectin system has been shown to play an important role in regulating vascular inflammation in man, cats, pigs, cows and mice, there is a paucity of information about equine P-selectin.

As monocytes are central to a number of pathophysiological responses caused by the overproduction of immune mediators, modulation of leucocyte adhesion has been a goal for the design of many therapeutics. Anti-P-selectin therapy has shown a great deal of potential for the amelioration of a variety of these disease states. One of the most promising P-selectin agonists, rPSGL1-Ig, is a recombinant, soluble form of PSGL-1 truncated after the P-selectin binding domain and fused to the Fc region of a human immunoglobulin (Ig). As this molecule has been shown to prevent inflammation in a number of primary and secondary vascular disorders (eg myocardial infarcts and ischaemia/reperfusion injury, respectively), we hypothesised that rPSGL1-Ig can block both signalling events and release of chemotactic cytokines in human monocytes in an in vitro setting. Indeed, we have seen that rPSGL1-Ig can block the PSGL-1 mediated activation of the mitogen activated protein kinases (MAPKs) which has been implicated in interleukin-8 (II-8) release in neutrophils. As such, we examined cytokine production in monocytes induced both by PSGL-1 ligation and by activated platelets. We have found rPSGL1-Ig to be effective in blocking both II-8 and monocyte chemotactic protein 1 (MCP-1) production, suggesting that activated platelets bind to monocytes and induce signalling events central to immune mediator biosynthesis. Our results demonstrate the efficacy of this drug in modulating inflammatory reactions.

Ascending colon volvulus (ACV) of 4 to 6 h results in the adhesion of neutrophils to the injured endothelium, release of inflammatory mediators and loss of fluid, thrombosis of vessels and necrosis of the tissue (Chang 1992; Welbourn and Young 1992). P-selectin is an adhesion molecule that mediates rapid interactions between leucocytes and endothelial cells called ‘rolling’ (the ‘early phase’ of adhesion; Darien et al. 1991, 1993). This transient contact is a pre-requisite for firm adhesion (‘late phase’ of adhesion), extravasation of PMNs (inflammation), and tissue injury (Darien et al. 1995; Lukacs et al. 1995). P-selectin is stored in platelets and endothelial cells and expressed on their surface in response to various stimuli, such as endotoxin (Lorant et al. 1993; Granger and Kubes 1994; Diacovo et al. 1996). Most recently, studies using P-selectin blocking antibodies or using the human recombinant P-selectin ligand. P-selectin glycoprotein ligand-1 (PSGL-1) fused to an IgG1 Fc molecule (referred to as the chimera rPSGL1-Ig) have been shown to prevent ischaemia/reperfusion injury (Elstaad et al. 1995; Lorant et al. 1995; Mazzone et al. 1993; Weyrich et al. 1995). In the author’s laboratory, it has been
shown that rPSGL1-Ig prevents PSGL-1-mediated signal transduction and blocks IL-8 and MCP-1 biosynthesis. Given the high incidence of life threatening colic and inflammatory bowel disease in the horse, and the importance of the P-selectin system in other species, we believe the P-selectin system should be investigated in the horse.

REFERENCES


Non-infective arthritis is an extremely common problem in the horse. It is nearly always considered traumatic in origin with a spectrum of injury ranging from traumatic synovitis and capsulitis through ligamentous injury, meniscal injury, osteochondral fracture, subchondral bone disease, to osteoarthritis (progressive degradation of articular cartilage; McIlwraith 1996). Although examination of the synovial membrane in all these entities will reveal accumulation of lymphocytes and plasma cells as a common reaction, it has been found that this is a non-specific reaction and does not imply an immune basis (McIlwraith 1996).

Rheumatoid arthritis has never been described in the horse but isolated cases of lupus erythematosus have been described.

On the other hand, considerable work in the last 10 years has been undertaken on the molecular basis of joint disease in the horse; and cytokines and inflammatory mediators have been identified. Inflammatory mediators associated with destruction of hyaluronan (HA) in synovial fluid as well as articular cartilage degradation include:

1) the cytokines interleukin-1 and TNF-alpha;
2) metalloproteinases and ‘aggrecanase’;
3) prostaglandins E₂; and
4) free radicals.

Metalloproteinases are considered to play a major role in the degradation of the extracellular matrix. Matrix metalloproteinases that have been incriminated in osteoarthritis include collagenase 1 (MMP-1), collagenase 2 (MMP-8), collagenase 3 (MMP-13), stromelysin 1 (MMP-3), and 2 gelatinases (MMP-2 and MMP-9; Caron et al. 1992; McIlwraith 1996; Clegg et al. 1997a,b). Evidence for varying roles of these MMPs in equine articular cartilage degradation is accumulating. Up-regulation of MMP-1 has been demonstrated with in situ hybridisation studies on equine synovial membrane and cartilage in diseased equine joints in our laboratory. On the other hand, recent evidence accumulated in both human and equine studies indicate that the primary collagenase involved in the degradation of type II collagen of articular cartilage may, in fact, be collagenase 3 (MMP-13) (Caron et al. 1992).

Consideration of the breakdown of proteoglycan is focused on stromelysin (also called proteoglycanase or MMP-3) as well as an enzyme ‘aggrecanase’ recently identified and classified as an ADAMTS. Stromelysin cleaves proteoglycans between the G1 and G2 domain of aggrecan at the asparagine (341) - phenylalanine (342) bond (Flannery et al. 1992). It has a wide variety of substrates including the various proteoglycans as well as type II collagen in the non-helical site and molecular cloning and cartilage gene expression of equine stromelysin I (MMP-3) has been described by Balkman and Nixon (1998). In addition to cleavage of aggrecan at the MMP site, cleavage between the G1 and G2 domains also occurs at the GLU373-ALA374 site and this has been attributed to aggrecanase (Sande et al. 1991). Equine matrix metalloproteinases 2 and 9 have been characterised in the horse (Clegg et al. 1997a,b) and can cause further cleavage to the 1/4 and 3/4 fragments of type II collagen generated by collagenase cleavage. All metalloproteinase is secreted as latent proenzymes that are activated extracellularly. Metalloproteinases are inhibited by 2 tissue inhibitors of metalloproteinase known commonly as TIMP (TIMP-1 and TIMP-2) (McIlwraith 1996). Pioneering work with equine metalloproteinases has been done in separate laboratories (Caron et al. 1996; Clegg et al. 1997a,b; Richardson and Dodge 1997; Balkman...
and Nixon 1998) with collagenase -3 and stromelysin sequences cloned (Caron et al. 1996; Balkman and Nixon 1998). Metalloproteinases as well as PGE$_2$ and NO have also been involved in other joint conditions such as subchondral cystic lesions in horses. It was demonstrated that the fibrous tissue of subchondral cystic lesions released NO, PGE$_2$ and neutral MMPs into the culture media suggesting that these may be partially responsible for the maintenance, slow healing rate and expansion of these lesions (Von Rechenberg et al. 2000). Later work has shown active bone resorption by the fibrous tissue of subchondral cystic lesions.

Prostaglandins (primarily E group) were produced in inflamed joints and can cause a decrease in the proteoglycan content of the cartilage matrix. The presence of PGE$_2$ and synovial fluid from inflamed joints has been demonstrated in the horse and PGE$_2$ is used as an objective index of the level of synovitis in the author’s laboratory (Frisbie et al. 1997; Kawcak et al. 1997). Oxygen-derived free radicals including superoxide anion, hydroxyl radicals and hydrogen peroxide may be released from injured joint tissues and their increase in the synovial fluid of cases of equine joint disease have been demonstrated (Dimock et al. 2000).

Interleukin-1-like activity was first demonstrated in inflamed equine joints by Morris et al. (1990). An equine IL-1 containing extract was produced by May et al. (1990) and the ability of this extract to degrade equine cartilage matrix demonstrated. Billinghamurst et al. (1995) first demonstrated induction of intra-articular TNF during acute inflammatory responses in equine arthritis. Earlier it had been demonstrated that recombinant human TNF-alpha, like IL-1, causes cartilage degradation (Saklatvala 1986). It was presumed due to stimulating the chondrocyte to produce matrix-degrading enzymes (Bunning and Russell 1989). However the role of TNF in individual clinical arthritides remains unclear. Based on inhibition studies it appears that IL-1 is the principal cytokine responsible for articular cartilage degradation and TNF-alpha contributes more to clinical morbidity and pain. IL-1 and TNF-alpha have been demonstrated using RT-PCR in the synovial membrane of inflamed equine joints. Interleukin-1 acts through receptors on chondrocytes causing release of MMPs and PGE$_2$ and these findings are now being incorporated into the development of biological therapies. The complete gene sequences for equine IL-1 alpha, IL-1 beta and IL-1 receptor antagonist were described by Howard et al. (1998a,b). There are 2 types of TNF (P55 and P75). Both have been identified in synovial tissue and greater numbers of receptors are seen in joints affected by RA in comparison to OA synovial tissue (Deleuran et al. 1992). These receptors can be shed and act as soluble inhibitors of TNF (TNF binding proteins). Increased serum concentrations of soluble TNF receptors (sTNF-R) have been detected in patients with RA and OA in comparison to healthy controls (Cope et al. 1992).

Traditionally the treatment of equine joint disease has been based on non-steroidal anti-inflammatory drugs and corticosteroids (ie symptomatic suppression of inflammation). Such anti-inflammatory therapy does have biological benefits in some instances as demonstrated with triamcinolone acetonide by Frisbie et al. (1997). Other novel therapies have included the use of intravenous hyaluronan (Kawcak et al. 1997).

More recently, biological therapies aimed at specifically addressing critical mediators have been investigated. The use of gene therapy with an adenoviral-IRAP vector has proven quite successful. The equine IL-1 receptor antagonist (IRAP) sequence defined by Howard et al. (1998b) was used. Good transduction was demonstrated in vitro and in vivo and a single injection of adeno-IRAP markedly suppressed experimental osteoarthritis in the horse (Frisbie and McIlwraith 2000). Challenges remain with this therapy as repeat dosing is not possible with the current vector combination. A clinical trial is about to commence in horses. The use of MMP inhibitors has also been evaluated. In vitro work by R.C. Billinghamurst (New York Academy of Science) implies that the inhibitor BAY 12-9566 apparently inhibits equine aggregcanase as well as MMPs, at least implied by its inhibition of IL-1 mediated degradation of articular cartilage. This material is currently being tested in an in vivo canine model of osteoarthritis and a clinical trial in the horse is being planned. Other biological methods are potentially capable of treating equine joint disease, including promotion of beneficial cytokines or inhibition of deleterious ones. In addition to IL-1 receptor antagonist, techniques with TNF soluble receptors IL-4 and IL-10 deserve attention. Beneficial effects from anabolic cytokines such as IGF-1 in cartilage healing have also been implied with in vitro work, and gene therapy with IGF-1 is currently being investigated.
REFERENCES


Articular cartilage is an avascular, aneural tissue which covers the articulating surfaces of bones. It functions to distribute load and minimise friction associated with weight-bearing and joint movement. Articular cartilage is relatively acellular, with chondrocytes comprising only 1–2% of the biomass. These cells, however, are responsible for the synthesis and maintenance of the extracellular matrix which constitutes the bulk of cartilaginous tissues. It is the molecular components and organisation of this extracellular matrix which is responsible for the specialised biomechanical and functional properties of articular cartilage. In osteoarthritis, structural matrix integrity is compromised leading to articular cartilage degeneration and a progressive loss of joint function. We have investigated how synovitis and corticosteroid therapy affect cartilage matrix protein synthesis by articular chondrocytes, testing the hypothesis that changes in the expression of specific matrix proteins contribute significantly to molecular mechanisms that link synovitis and corticosteroid therapy to long-term degenerative changes in articular cartilage.

Comparing gene expression in articular cartilage between normal and mildly inflamed equine carpi, it is evident that chondrocytes respond to acute joint inflammation by increasing the synthesis of matrix proteins. Although mild synovitis of 8 days’ duration did not result in any detectable histopathological changes in the articular cartilage, steady state type II procollagen mRNA increased 5-fold and aggrecan mRNA 2-fold. This anabolic response of chondrocytes to synovitis probably reflects compensatory matrix protein synthesis in order to maintain cartilage homeostasis. Joint inflammation elevates expression of matrix proteases. In response to the resulting increase in the rate of matrix protein degradation, the upregulation of matrix protein synthesis by chondrocytes attempts to maintain an overall balance and structural matrix integrity.

Corticosteroids are potent anti-inflammatory agents used widely for the therapeutic management of both acute and chronic arthritic diseases. Although these agents reduce expression of matrix metalloproteinases, and are clearly helpful in relieving the symptoms of joint inflammation, experimental evidence suggests that corticosteroids damage articular cartilage independent of the disease process. Pathological changes described in articular cartilage of previously normal joints following intra-articular methylprednisolone acetate (MPA) or betamethasone administration include: loss of basophilia and decreased safranin O staining intensity, chondrocyte necrosis and hypocellularity, decreased proteoglycan content, decreased collagen synthesis, increased water content, and delayed healing of experimentally-induced osteochondral defects. These degenerative changes alter the composition of articular cartilage and appear to render the tissue more susceptible to mechanical injury both in vitro and in vivo. The mechanism of the steroid injury, however, remains unclear.

For in vivo studies, the carpi of young adult ponies were injected with MPA at 0.1 mg/kg and articular cartilage collected 24 h later. For in vitro studies, non-calcified full-thickness shavings of articular cartilage were collected from weight-bearing areas of the stifle, shoulder, elbow and carpal joints of horses 18–24 months of age. These joints had no gross evidence of osteoarthritis or other joint abnormality. The extracellular matrix was removed by an overnight collagenase digestion and articular chondrocytes isolated. Chondrocytes used for total RNA isolation were
seeded in 75 cm² culture flasks at an initial density of $5 \times 10^6$ cells/flask (6.7 $\times 10^4$ cells/cm²). Chondrocytes used to assess methylprednisolone-induced cytotoxicity were seeded in 962 mm² (6 well) culture plates at a density of $2.1 \times 10^4$ cells/cm². The cultures were maintained in Ham’s F12 medium supplemented with 10% (v/v) FBS, L-ascorbic acid (50 µg/ml), L-glutamine (300 µg/ml), $\alpha$-ketoglutaric acid (30 µg/ml), HEPES (125 mM), penicillin (20 units/ml), and streptomycin (20 µg/ml). Changes in gene expression were assessed by gel electrophoresis of total chondrocyte RNA, followed by Northern blot hybridisation using equine-specific cDNA probes.

**In vivo** steady-state mRNA levels of type II procollagen and aggregcan core protein were suppressed 6-fold and 2-fold, respectively, by a single intra-articular injection of MPA. **In vitro**, type II procollagen expression was suppressed in articular chondrocytes by corticosteroids in a dose-dependent fashion as medium methylprednisolone concentrations were increased from $1 \times 10^1$ to $1 \times 10^8$ pg/ml. Steady-state levels of type II procollagen mRNA decreased to less than 10% of control values at medium methylprednisolone concentrations $\geq 10^5$ pg/ml ($\geq 0.2$ µM). A parallel change in type I procollagen ($\alpha2$) mRNA did not occur, with low levels of expression remaining relatively stable in all samples. Cytotoxicity was observed as methylprednisolone levels were increased further from $1 \times 10^8$ to $1 \times 10^9$ pg/ml.

The clinical significance and pathogenesis of detrimental corticosteroid effects on articular cartilage, so-called steroid-induced arthropathy, is debated. Suppression of phenotypic markers of chondrocyte differentiation as it relates to matrix protein expression clearly has the theoretical potential to compromise the structural integrity of cartilage over time. Corticosteroid-induced chondrocyte necrosis, especially if superimposed on the loss of chondrocytes that occurs during the normal progression of osteoarthritis, would also exacerbate a degenerative process. As with any clinical intervention, however, detrimental side effects of intra-articular corticosteroids need to be assessed in the context of their therapeutic benefits that include suppression of matrix metalloproteinase activities and pain relief. The issue of symptomatic pain relief is further complicated by its potential to encourage premature overuse of a structurally and metabolically compromised joint. Knowledge of how synovitis and corticosteroids (independently and in combination) affect chondrocyte function and matrix protein synthesis is relevant to understanding the pathogenesis of osteoarthritis and improving therapeutic strategies for joint inflammation.
Osteoarthritis (OA) is a well known problem for athletic horses. The pathophysiological hallmark of the disease is a chondrocyte-mediated digestion and progressive loss of important cartilage matrix components. Interleukin-1 (IL-1) and other proinflammatory mediators promote the degradation of the matrix by inducing the synthesis of proteolytic enzymes, the most important of which are believed to be the matrix metalloproteinases (MMPs). Another inflammatory mediator implicated in OA pathophysiology is nitric oxide (NO). Nitric oxide is hypothesised to contribute to a number of the deleterious influences of IL-1 on cartilage metabolism including augmented expression and activation of MMPs and reduced synthesis of the natural receptor antagonist protein for IL-1 (Murrell et al. 1995; Pelletier et al. 1996). In a canine OA model, treatment with a selective inhibitor of inducible nitric oxide synthase (iNOS), resulted in a significant reduction of arthritis lesions of cartilage and synovial membrane and significantly reduced MMP activity in cartilage extracts (Pelletier et al. 1999). These data suggest that inhibition of NO release may attenuate the progression of OA by reducing MMP synthesis in cartilage. The hypothesis tested in this study was that iNOS expression and/or activity of IL-1β-stimulated equine chondrocytes are influenced by commonly used anti-arthritis compounds.

RT-PCR methods were used to amplify a portion of the equine iNOS message to prepare an RNA probe. Northern blot analyses were used to quantify the expression of iNOS in first passage monolayer cultures of equine articular chondrocytes propagated in the presence or absence of recombinant equine interleukin-1β (reIL-1β), dexamethasone, (10⁻⁶ M and 10⁻⁵ M), and polysulphated glycosaminoglycan (PSGAG), hyaluronan, and phenylbutazone, each at concentrations of 10 and 100 µg/ml. Nitrite concentrations in conditioned media of similarly treated cells were used to quantify iNOS activity indirectly. Comparison of means for relative expression of iNOS as well as media concentrations of nitrite were performed using a 2-way ANOVA (blocked by horse) followed by a Dunnett’s test. A P value of <0.05 was considered significant.

A 497-bp fragment of equine iNOS cDNA, amplified by use of RT-PCR, had approximately 88% sequence identity with the human cDNA sequence and 92% identity of the deduced amino acid sequence indicating that the cloned fragment is a portion of the corresponding equine iNOS message. Recombinant equine IL-1β increased the expression of iNOS in a dose-dependent manner. This result was paralleled by increased concentrations of nitrite in the culture media of reIL-1β-treated cells. Dexamethasone and PSGAG significantly reduced iNOS gene expression and conditioned media nitrite concentrations in cytokine-stimulated cultures (Figs 1 and 2). Hyaluronan and phenylbutazone had no statistically significant effect on the expression or activity of iNOS.

Although the role of NO in the physiology and pathobiology of equine vascular reactivity and neuromuscular transmission in a number of organ systems has been the subject of recent research, its role in equine OA pathogenesis has received little study. We prepared an RNA probe to characterise an inducible member of the NOS family that corresponds to a similarly-sized cDNA demonstrated in cytokine-stimulated human chondrocytes. Results of subsequent experiments using reIL-1β-stimulated cultures complement previous reports describing NO synthesis by equine chondrocytes in response to stimulation.
Fig 1: Effects of anti-arthritis preparations on reIL-1β stimulated iNOS expression by normal chondrocytes. Treatments include negative and positive (IL-1β), controls and treatments of dexamethasone, polysulphated glycosaminoglycan (polysulphated GAG), hyaluronan and phenylbutazone. IL-1-stimulated control and treated cultures have cross-hatched bars and non-stimulated controls have clear bars. Inducible NOS expression was calculated as the ratio of the intensity of the iNOS bands to the intensity of the ethidium bromide-stained 28S ribosomal RNA band of the electrophoretic gel. Values are mean (± se) expression from 3 experiments. Relative expression of reIL-1b-stimulated iNOS in chondrocytes treated with dexamethasone (10^{-6}M and 10^{-5}M) and polysulphated glycosaminoglycan (10 µg/ml and 100 µg/ml) (marked by asterisks) was significantly (P<0.05) less than that of reIL-1β-stimulated (positive control) cells.

Fig 2: Effects of anti-arthritis preparations on reIL-1β stimulated iNOS activity by normal chondrocytes. Treatments include negative and positive (IL-1β), controls and treatments of dexamethasone, polysulphated glycosaminoglycan (polysulphated GAG), hyaluronan and phenylbutazone. IL-1-stimulated control and treated cultures have cross-hatched bars and non-stimulated controls have clear bars. Inducible NOS activity was determined indirectly using nitrite concentrations using the Greiss reaction in conditioned media following a 24 h incubation. Values are mean (± se) expression from 3 experiments. Inducible NOS activity of reIL-1β-stimulated iNOS in chondrocytes treated with dexamethasone (10^{-6}M and 10^{-5}M) and polysulphated glycosaminoglycan (10 µg/ml and 100 µg/ml) (marked by asterisks) was significantly (P<0.05) less than that of reIL-1β-stimulated (positive control) cells.
with proinflammatory mediators. We also provide data indicating that chondrocyte iNOS expression and activity are influenced by corticosteroids and polysulphated glycosaminoglycans.

Northern blot analyses indicated that expression of our chondrocyte iNOS was inhibited by dexamethasone at 10^{-6}–10^{-5}\ M. In other species, a number of isoforms of NOS have been described and vary in their sensitivity to glucocorticoids (Palmer \textit{et al.} 1992, 1993). Many iNOS isolated from mammalian chondrocytes are insensitive to corticosteroids at modest doses. However, parallelling our findings, bovine iNOS expression is inhibited with high doses of dexamethasone (Murrell \textit{et al.} 1996). These findings may suggest a limited influence of endogenous glucocorticoids on iNOS expression but that pharmacological doses are inhibitory.

PSGAG was observed to reduce rIL-1β-induced expression of iNOS. Although polysulphated glycosaminoglycans are generally considered effective for the symptomatic treatment of OA in man and animals, their mechanism(s) of action in pain relief and disease modification (chondroprotection) remain to be confirmed. Based on our results, it is possible that, in addition to other potential effects, the chondroprotective potential of PSGAG may be due to their ability to inhibit the expression of iNOS. It has been shown that heparinoids, molecules of similar structure to polysulphated glycosaminoglycan, are capable of influencing gene expression in connective tissue mesenchyme. One recent report indicates that heparin down-regulates MMP expression in human gingival fibroblasts (Gogly \textit{et al.} 1998) and it has been shown that heparin and pentosan polysulphate, a sulphated glycosaminoglycan of plant origin, down-regulate phorbol-induced cancer cell proliferation by interfering with the binding of transcription factor to gene promoter AP-1 sites (Busch \textit{et al.} 1992).

NO is considered an important mediator in the pathophysiological processes of arthritis and an inducible NOS is expressed by equine chondrocytes. Results of this study provide previously unreported data on the modulation of equine iNOS expression and activity. These data support a role for NO in the pathogenesis of equine OA, and suggest that pre-translational regulation of the iNOS gene by dexamethasone and polysulphated glycosaminoglycans appears to contribute to the cartilage-sparing properties of these compounds.

\textbf{REFERENCES}


# LIST OF PARTICIPANTS

<table>
<thead>
<tr>
<th>Name</th>
<th>Nationality</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorothy Ainsworth</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>Doug Antczak</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>Cormac Breathnac</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>John Caron</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>Laurent Couëtil</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>Benjamin Darien</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>Julia Flaminio</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>Steeve Giguère</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>Gittan Gröndahl</td>
<td>Sweden</td>
<td></td>
</tr>
<tr>
<td>Duncan Hannant</td>
<td>United Kingdom</td>
<td></td>
</tr>
<tr>
<td>Steve Hines</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>Mark Holmes</td>
<td>United Kingdom</td>
<td></td>
</tr>
<tr>
<td>David Horohov</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>Tom Klei</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>Julia Kydd</td>
<td>United Kingdom</td>
<td></td>
</tr>
<tr>
<td>Jean-Pierre Lavoie</td>
<td>Canada</td>
<td></td>
</tr>
<tr>
<td>Dr Paul Lunn</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>Jamie MacLeod</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>Bruce McCorum</td>
<td>United Kingdom</td>
<td></td>
</tr>
<tr>
<td>Travis McGuire</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>Wayne McIlwraith</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>James Moore</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>Rustin Moore</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>Lesley Nicolson</td>
<td>United Kingdom</td>
<td></td>
</tr>
<tr>
<td>Gene Pranzo</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>Sharanne Raidal</td>
<td>Australia</td>
<td></td>
</tr>
<tr>
<td>Ed Robinson</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>Elisa Santschi</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>Josh Slater</td>
<td>United Kingdom</td>
<td></td>
</tr>
<tr>
<td>Gisela Soboll</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>Jeff Stott</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>John Timoney</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>Laurent Viel</td>
<td>Canada</td>
<td></td>
</tr>
<tr>
<td>Jan Wade</td>
<td>United Kingdom</td>
<td></td>
</tr>
<tr>
<td>Bettina Wagner</td>
<td>Germany</td>
<td></td>
</tr>
<tr>
<td>Joe Watson</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>Eva Wattrang</td>
<td>Sweden</td>
<td></td>
</tr>
<tr>
<td>David Wilson</td>
<td>United States</td>
<td></td>
</tr>
</tbody>
</table>
AUTHOR INDEX

AINSWORTH, D.M. et al., 84
ALLEN, G.P. see BREATHNACH, C.C. et al.; KYDD, J.H. et al.
ANTCZAK, D.F., 29 and see AINSWORTH, D.M. et al.
APPLETON, J.A. see AINSWORTH, D.M. et al.
ARGYLE, D. see NICOLSON, L. et al.
AVIZA, G.A. see AINSWORTH, D.M. et al.

BEATTIE, C.W. see SANTSCHI, E.M. et al.
BRASIL, T. see MCGORUM, B. and BRAZIL, T.
BREATHNACH, C.C. et al., 26
CARON, J.P. see TUNG, J.T. et al.
COUËTIL, L.L. et al., 86

DARIEN, B.J., 91
EADES, S.C. see HOLM, A.S. et al.

FLAMINIO, M.J.B.F. et al., 69 and see RUSH, B.R. et al.
FRASER, D.G. see McGUIRE, T.C. et al.
FUXTLER, L. see WATTRANG, E. et al.

GIGUÈRE, S., 11; 59
GRÖNDAHL, G., 46
HANNANT, D., 19 and see WATTRANG, E. et al.; KYDD, J.H. et al.
HINES, M. see HINES, S. et al.
HINES, S. et al., 42
HOLLIMAN, A. see HOLMES, M.A. et al.
HOLM, A.S. et al., 89
HOLMES, M.A. et al., 35
HOPKINS, C. see NICOLSON, L. et al.
HOROHOV, D.W., 7; 54 and see LAVOIE, J-P. and HOROHOV, D.W.; SOBOLL, G. et al.
HUNT, M.A. see COUËTIL, L.L. et al.

JACKSON, K.A. see WATSON, J.L. et al.
JESSETT, D.M. see WATTRANG, E. et al.
KLEI, T.R., 17
KYDD, J.H. et al., 65

LAVOIE, J-P. and HOROHOV, D.W., 82
LEIB, S.R. see McGUIRE, T.C. et al.
LUHM, D.P., 41 and also SOBOLL, G. et al.

MACLEOD, J., 97
McGUIRE, T.C. et al., 3 and see HINES, S. et al.
MCGORUM, B. and BRAZIL, T., 79
MCLWRAITH, C.W., 94
McMONAGLE, L. see NICOLSON, L. et al.
MARTI, E., 33
MAY, P.D.F. see HOLMES, M.A. et al.
MEALEY, R.H. see McGUIRE, T.C. et al.
MOORE, J.N., 87
MOORE, R.M. see HOLM, A.S. et al.
NICOLSON, L. et al., 57
NORTON, L. see HINES, S. et al.

OLIVER, J.L. see HOLM, A.S. et al.
OLSEN, C.W. see SOBOLL, G. et al.
O’NEILL, T. see KYDD, J.H. et al.
ONIONS, D. see NICOLSON, L. et al.

PRIEUR, D.J. see McGUIRE, T.C. et al.
RAIDAL, S.L., 49
RIDGELY, S.L. see McGUIRE, T.C. et al.
RINK, A. see SANTSCHI, E.M. et al.

ROBINSON, N.E., 73

ROSSENTHAL, F.S. see COUËTIL, L.L. et al.

RUSH, B.R. et al., 67 and see FLAMINIO, M.J.B.F. et al.

SANDERS, L. see NICOLSON, L. et al.

SANTIAGO, M.A. see AINSWORTH, D.M. et al.

SANTSCHI, E.M. et al., 64

SCHIJNS, V. see NICOLSON, L. et al.

SCHOLES, S.F.E. see HOLMES, M.A. et al.

SCHOLTES, N. see NICOLSON, L. et al.

SHEORAN, A.S., see BREATHNACH, C.C. et al.; TIMONEY, J.F. and SHEORAN, A.S.

SHUMAN, W. see FLAMINIO, M.J.B.F. et al.

SLATER, J., 14

SOBOLL, G. et al., 23

STONE, D. see HINES, S. et al.

STOTT, J.L. see WATSON, J.L. et al.

TAYLOR, S. see NICOLSON, L. et al.

TIMONEY, J.F. and SHEORAN, A.S., 21

TUNG, J.T. et al., 99

Van KUILEKOM, H. see NICOLSON, L. et al.

VIEL, L., 77

VENUGOPAL, C.S. see HOLM, A.S. et al.

VENTA, P.J. see TUNG, J.T. et al.

WAGNER, B., 30

WATSON, J.L. et al., 44

WATTRANG, E. et al., 62 and see KYDD, J.H. et al.


YATES, P. see WATTRANG, E. et al.

YEARGEN, M.R. see BREATHNACH, C.C. et al.