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Innate immunity during Equid Herpesvirus 1 (EHV-1) infection

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SUMMARY

Intrinsic phagocytosis and killing of *C. albicans* by equine monocytes and polymorphonuclear leucocytes (PMN) was examined during Equid Herpesvirus 1 (EHV-1) (subtypes 1 or 2) and Adenovirus infections. Monocyte function increased during EHV-1 subtype 2 and Adenovirus infection. Conversely, there was an impairment of monocyte ingestion during EHV-1 subtype 1 infection which was ascribed to virus replication in peripheral blood mononuclear cells. PMN phagocytosis was not decreased in any of the infections studied. The raised levels of haemolytic complement in animals which subsequently developed EHV-1 subtype 1 induced paresis suggested an abnormality of complement turnover. Increased levels of interferon were evident in the nasal secretions of both subtype 1 and subtype 2 infected animals but only subtype 1 virus induced measurable levels of serum interferon. No intrinsic abnormality of interferon production by monocytes or lymphocytes was found.

Keywords monocytes polymorphonuclear leucocytes interferon complement Equid Herpesvirus-1

INTRODUCTION

Equid Herpesvirus-1 (EHV-1) is classified into two subtypes by growth characteristics in tissue culture (Burrows & Goodridge, 1973; Studdert & Blackney, 1979), cross-neutralization tests (Burrows & Goodridge, 1973), pathogenicity in mice (Patel & Edington, 1983), cellular tropism (Patel, Edington & Mumford 1982) and restriction endonuclease analysis (Sabine, Robertson & Whalley, 1981; Studdert, Simpson & Roizman 1981). Both subtypes produce rhinopneumonitis, but only subtype 1 isolates consistently produce abortion (Manninger & Czontos 1941; Burrows & Goodridge 1973) and paresis (Manninger, 1949; Saxegaard, 1966; Mumford & Edington, 1980).

Upon challenge with plaque-cloned 'paresis' isolates of EHV-1 not all animals develop ataxia, despite appearing to possess similar pre-exposure levels of immunity (Edington, Bridges & Patel 1986). In view of the predilection of EHV-1 paresis isolates for vascular endothelial cells, in particular those of the central nervous system (CNS) (Patel *et al.*, 1982), it was reasoned that immune mechanisms which prevent virus dissemination could be abnormal in paretic animals. This has led us to initiate a programme for analysing various parameters of the equine immune response with the aim of revealing underlying immune dysfunction and thereby identifying those animals at risk from EHV-1 infection.

Whilst host opposition to virus reinfection must be largely dependent on the presence of neutralizing antibody and immune T cells, the containment of virus during the early stages of primary infection is necessarily reliant on a versatile non-specific immune response. Interferon, complement, natural killer cells and phagocytes are all implicated in the prevention of virus

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replication and spread (Jarstrand & Einhorn 1981; Grewal & Babiuk 1980; Starr & Garrabrant 1981; Morse & Morahan 1981), a process which allows sufficient time for the host to develop a virus specific immune response. In this paper we present information about interferon, complement and intrinsic phagocyte function during experimental infection with the two EHV-1 subtypes.

MATERIALS AND METHODS

Animals, viruses and experimental infection. All animals were inoculated with 1·0 ml of fluid given by an intranasal swab and by nebuliser.

Group 1. Six Welsh mares (M1–6) all at least 6 years old, were inoculated with a $10^{5.5}$ tissue culture infective dose ($TCID_{50}$)/ml suspension of EHV-1 subtype 1 originally isolated from a paraplegic gelding.

Group 2. Five yearling Welsh ponies (Y1–5) were inoculated with a $10^{5.3}$ $TCID_{50}$/ml suspension of EHV-1 subtype 1 originally isolated from the spleen of a paraplegic horse during an outbreak of EHV-1 abortion (Mumford & Edington 1980).

Group 3. Five yearling Welsh ponies (Y6–10) were inoculated with a $10^{5.5}$ $TCID_{50}$/ml suspension of EHV-1 subtype 2 originally isolated from the upper respitratory tract of a horse during an outbreak of respiratory disease.

Group 4. A control group of five yearling Welsh ponies (Y11–15) were inoculated with a $10^{5.5}$ $TCID_{50}$/ml suspension of Equine Adenovirus originally isolated from a case of Cauda Equina Neuritis (Edington *et al.*, 1984).

Concurrent field infection was rule out by serological monitoring for a minimum period of 3 weeks preceding experimental inoculation. Animals were kept in isolation from other horses.

Each experiment included two additional animals which were uninfected, kept in isolation, and provided leucocytes and sera for internal controls. Where possible the values for internal controls were compared to normal ranges derived from uninfected yearlings. Results were discarded from those tests in which the internal controls differed significantly from the normal range.

Clinical examination included daily rectal temperatures and observation at exercise.

Preparation of leucocytes. Heparinised (preservative free, 10 u/ml) venous blood was allowed to stand at room temperature for 30–40 min in order to sediment red cells. The leucocyte rich upper layer was then removed, diluted with an equal volume of heparinised (10 u/ml) Dulbecco's phosphate buffered saline (PBS) pH 7·2, and floated by underlaying a half volume of 'Lymphoprep' (Nyegaard UK Ltd). These preparations were then centrifuged at 800 *g*/15 min/20°C. The resulting fractions of peripheral blood mononuclear leucocytes (MNCs), or polymorphonuclear leucocytes (PMNs), were each removed and washed three times in PBS by 250 *g* centrifugation (7 min/4°C). After the last wash cells were resuspended in media and at concentrations appropriate to their destined assay.

Cell suspensions had viabilities >96% as evidence by trypan blue dye exclusion or acridine orange-ethidium bromide fluorescence. PMN cell suspensions were not less than 95% pure whilst MNC fractions, containing monocytes identified by non-specific esterase (Horwitz *et al.*, 1977) showed <3% contamination with PMNs. MNC fractions contained 7–12% monocytes, the rest being comprised of lymphocytes.

Virus isolation and complement fixing antibody. Virus isolation was performed according to Patel *et al.* (1982).

Virus isolation from nasal swabs: swabs were washed into 2·0 ml volumes of transport medium (BSA 1% w/v in PBS pH 7·2 containing penicillin 700 u/ml, streptomycin 7 μg/ml and 'Fungizone' 11 μg/ml and left to stand for 30 min/20°C. Subsequently, 100 μl aliquots were delivered, in triplicate, to 16 mm diameter tissue culture plate wells containing confluent monolayers of low passage (< 10) equine embryonic kidney (EEK) or established rabbit kidney (RK13) cells in 1·0 ml volumes of medium. The medium was 15 mM bicarbonate buffered Eagle's minimal essential medium (MEM) supplemented with 20 mM L-glutamine and non-essential amino acids, either 5% newborn calf serum (RK13s) or 10% fetal calf serum (EEKs), and antibiotics (penicillin 200 u/ml, streptomycin 2 μg/ml and 'Fungizone' 4 μg/ml).

Virus isolation from purified peripheral blood leucocytes: 10^6 washed cells were delivered to

indicator cells as described for nasal swabs. In both instances virus adsorption was allowed to proceed for 2 h/37°C in a humidified 5% CO_2 in air atmosphere. The cell monolayers were then given two washes with equal volumes of PBS, the last wash replaced with 1·0 ml medium and the plates reincubated for up to 7 days before discard. Monolayers were examined daily for EHV-1 induced cytopathic effect; virus identity was determined from the growth of subtype 1, but not subtype 2, on RK13 cells (Burrows & Goodridge, 1973) and EHV-2 ruled out by indirect immunofluorescence of cytocentrifuged material from infected wells using hyperimmune rabbit, or convalescent foal, antiserum (Patel & Edington, 1983).

Complement fixing antibody to EHV-1 was measured according to Thomson *et al.* (1976). Antibody to adenovirus was measured by haemagglutination inhibition (Rose, 1969) using human type O erythrocytes.

Intrinsic phagocytosis and killing by monocytes and PMNs. A published radiometric method (Bridges *et al.*, 1980) was modified to investigate intrinsic function of equine phagocytes: two sets of sextuplet mixtures containing 2×10^5 *C. albicans* blastospores and 3×10^4 monocytes or PMNs in 100 μl 5% normal equine serum (NES) (v/v in 20 mM HEPES buffered RPMI-1640) were placed in round-based microtitre plate wells. Control mixtures, identical but for the absence of phagocytes, were also prepared in two sets of sextuplet. Measurement of monocyte function required extra mixtures, consisting of mononuclear cells in 5% NES-medium, to correct for leucocyte incorporation of radiolabel. Microtitre plate preparations were incubated for 40 min/37°C after which each well received 0·2 μCi ^{3}H-uridine (TRK 410, Amersham International). Then, for measurement of phagocytosis, mixtures of the first set were made up to 200 μl medium. Evaluation of killing was achieved by immediately lysing the phagocytes in the remaining set with 100 μl 0·6% w/v sodium deoxycholate and 100 μg/ml DNAase in 50% medium. Both sets were subsequently reincubated for 1 h/37°C to allow radiolabel incorporation by blastospores. Finally, cell associated radioactivity was collected onto glass fibre discs, dried, placed in scintillation fluid and counted in a β scintillation counter.

Results are expressed as phagocytic or killing indices (PI or KI respectively), a revised phagocytic index (RPI), or killing efficiency (KE) and were calculated by the following formulae:

$$\text{PI or KI} = \left[1 - \frac{\text{ct/min blastospores + phagocytes}}{\text{ct/min blastospores alone}}\right] \times 5$$

$$\text{RPI} = \frac{\text{PI test animal}}{\text{PI control animal}}$$

$$\text{KE} = \frac{\text{PI}}{\text{KI}} \times 100$$

Interferon assay. Antiviral activity present in serum or nasal secretions was measured as described by Yilma, McGuire and Perryman (1982) with the exception that Semliki virus was used instead of Vesicular stomatitis virus for the plaque reduction tests. Equine embryonic nasal mucosa cells in microtitre plate wells were preincubated (16–18 h/ 36°C) with putative interferon (IFN) samples, exposed to virus (10^2 plaque-forming units (pfu)/25 μl) and then overlaid with 50 μl carboxymethyl cellulose (CMC) (0·75% w/v in medium). After 48 h the indicator cells were fixed and stained (20 min/20°C) using 4% v/v formalin-PBS containing 0·1% w/v crystal violet. IFN units were expressed in terms of human IFN international units as no standard exists for equine IFN. Unstimulated and stimulated phytohaemagglutinin (PHA) 10 μg/ml or Equine influenza virus at a multiplicity of infection (m.o.i.) $>1·0$) mononuclear cells were similarly assayed for IFN production by harvesting supernatants from 24 h cultures ($3·0 \times 10^6$ MNCs/1·5 ml medium). These samples were also examined for plaque reduction of 10^2 pfu of EHV-1 subtype 1. Controls were a standard human α-interferon and an equine IFN made by harvesting the supernatants from control MNCs challenged *in vitro* with Equine Influenza virus.

Total haemolytic complement levels. Equine total haemolytic complement measurement was performed, with minor changes, according to Barta, Barta and Williams (1973). Modifications included using sensitised swine erythrocytes at a final concentration of 0·2% v/v, incubation of reaction mixtures for 30 min/37°C and the formulation of sucrose barbital buffer (SBB) diluent (6

mM barbital pH 9·0 containing 57 mM sucrose, 0·11 M NaCl, 1×10^{-3}M mg^{2+} and 3×10^{-4}M Ca^{2+}). duplicate 1·0 ml reaction mixtures contained final equine serum concentrations in the range 10–1%. Controls consisted of 'no lysis', '100% lysis' and serum only to correct for native colour. Indicator red cells were sensitized with rabbit antibody (Mayer, 1961). The CH_{50} titre was calculated from absorbance values at 413 nm using the von Krogh equation or Simpson's rule for finding the centroid of a curve.

Forty-five healthy ponies were used to construct the normal range: 10·5–35·5 CH_{50} units/ml serum. Results are expressed as per cent change $(100(x-y)/y)$ where x and y are CH_{50} values at consecutive time points.

RESULTS

Clinical observations and virus isolation

Following inoculation with EHV-1 subtype 1 or 2 animals became pyrexic (> 103°F; 39·4°C) while the degree of respiratory involvement ranged from a serous nasal discharge to dyspnoea and coughing. All horses were noticeably subdued and lethargic during the first week of infection.

None of the ponies in groups 3 and 4 were ataxic whereas a yearling (Y3), and three mares (M2,3,4), exposed to EHV-1 subtype 1 developed incoordination. This was most obvious whilst walking, particularly as they turned. Adenovirus infection (group 4) was not associated with any clinical abnormalities.

Figure 1 shows the pattern of virus recovery from nasal swabs and leucocytes. Virus was isolated

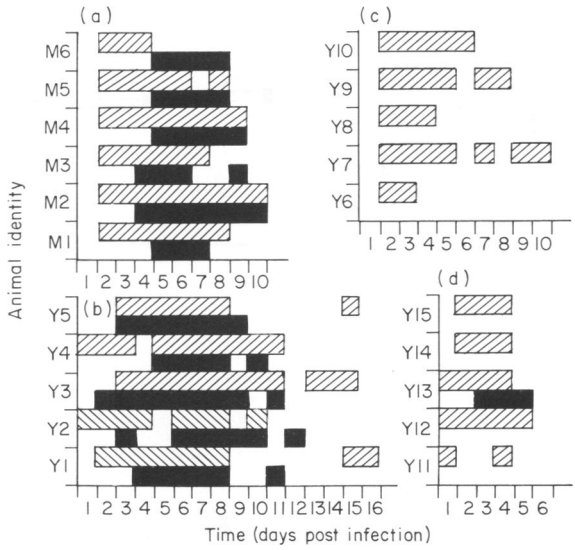

Fig. 1. Virus isolation from horses after experimental infection. (■, ▨) indicate virus recovery from peripheral blood leucocytes and nasal swabs respectively. Horse group identity: (a) EHV-1 subtype 1 infected mares; (b) EHV-1 subtype 1 infected yearlings; (c) EHV-1 subtype 2 infected yearlings and (d) equine adenovirus infected yearlings.

from the respiratory tract in all groups but only EHV-1 subtype 1 infected animals consistently developed viraemia. Duration of virus excretion from the respiratory tract was also greater in the subtype 1 infected yearlings (mean 9 days) when compared with subtype 2 (mean 4·6 days) or adenovirus (mean 3·4 days) infected ponies.

In all horses at least four-fold rise in complement fixing antibody titres to challenge virus were evident at 14 days after infection.

Intrinsic function of phagocytes

Monocyte intrinsic function, represented by the revised phagocytic index, of animals in all groups is shown in Fig. 2a. There was a significant increase in phagocytosis by MNPs from ponies infected with EHV-1 subtype 2 (days post-infection (dpi) 4–8, $0{\cdot}02 > P > 0{\cdot}01$), and from Adenovirus infected animals (dpi 3–7, $0{\cdot}05 > P > 0{\cdot}02$). Conversely, monocyte function in EHV-1 subtype 1 infected mares was decreased, albeit not significantly when compared to the normal resting range, but significantly when compared to the raised levels observed within group 3 and 4 animals ($P < 0{\cdot}001$). Monocyte ingestion by subtype 1 infected yearlings was also significantly different from group 3 and 4 animals ($P < 0{\cdot}001$).

The intrinsic phagocytic function of polymorphonuclear leucocytes was not significantly altered in any of the animals (Fig. 2b).

Examination of monocyte killing efficiency, during EHV-1 subtype 1 infection, revealed a significant fall at 9 dpi ($P < 0{\cdot}001$) whereas PMNs displayed enhanced killing between 4 and 14 dpi ($P < 0{\cdot}001$) (Fig. 3). The killing efficiency of mononuclear phagocytes (MNPs) during subtype 2 infection was not significantly reduced (Fig. 4). Unfortunately, technical difficulties prevented the assessment of PMN killing efficiency in subtype 2 infection but the KE of PMNs from adenovirus

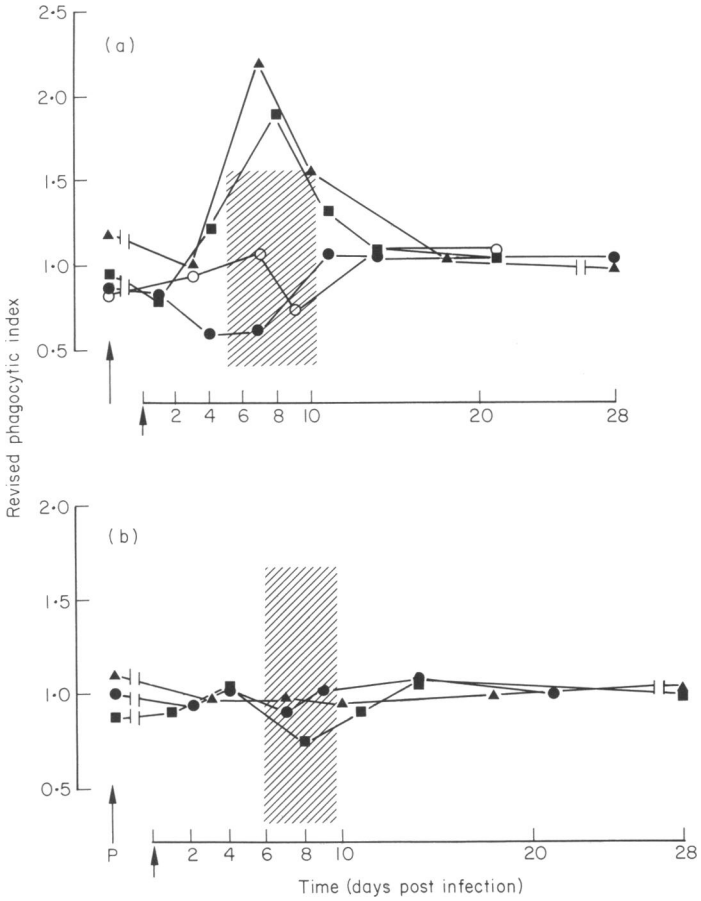

Fig. 2. Intrinsic function of phagocytes after experimental viral infection. Results expressed as the revised phagocytic index (RPI) versus time (days post infection). (■) indicates the normal ranges for RPI ($n = 25$), P indicates the preinfection value. Error bars have been left out for clarity. (a) Intrinsic ingestion by monocytes; (b) Intrinsic ingestion by PMNs. (●) EHV-1 subtype 1 infected mares; (○) subtype 1 infected yearlings; (■) EHV-1 subtype 2 infected yearlings; (▲) Adenovirus infected yearlings.

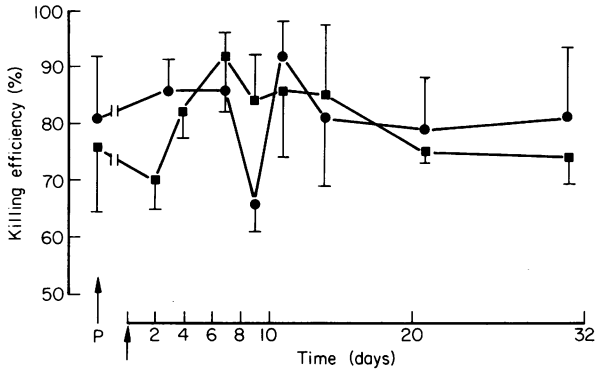

Fig. 3. Phagocyte killing efficiency (KE) during EHV-1 subtype 1 infection (group 2 yearlings). (●) monocyte KE; (■) PMN KE.

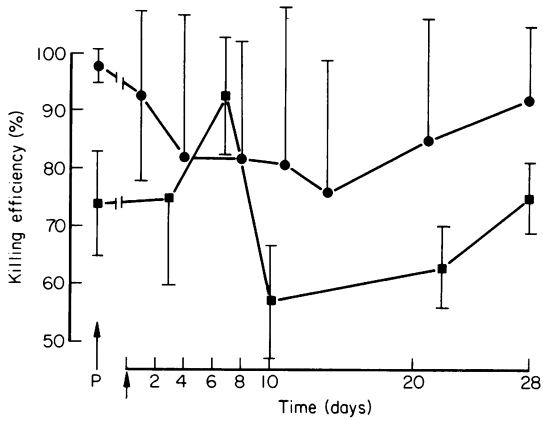

Fig. 4. Phagocyte killing efficiency (KE) during viral infection. (●) Monocyte KE during EHV-1 subtype 2 infection. (■) PMN KE during adenovirus infection.

infected horses also showed evidence of enhanced activity at 7dpi (Fig. 4). In addition the PMN KE of these ponies slumped at 10 dpi but was fully recovered by 28 dpi.

Preinfection examination of phagocytic function was not able to predict the identity of those animals which subsequently developed paresis, although it appeared that horses with most impairment of phagocytic function also had the most severe clinical symptoms. For example, the MNP PI for Y3, at 9 dpi, was 1·70 whereas unaffected animals in this group had a mean PI of $3{\cdot}0 \pm 0{\cdot}43$.

Interferon

Interferon levels were examined in EHV-1 subtype 1 infected mares and subtype 2 infected yearlings. Subtype 1 infection produced a biphasic increase in nasal IFN at 4 and 7 dpi as well as a rise in serum levels at 4 dpi. Subtype 2 infection did not induce measurable levels of IFN in serum but raised levels were found in nasal secretions (Fig. 5).

Monocytes or lymphocytes, irrespective of EHV-1 infection, always produced 100–1000 IFN units/ml when stimulated with Equine Influenza virus or PHA indicating the absence of any intrinsic defect of IFN production. Unstimulated mononuclear cells produced 20–100 IFN units/ 10^5 cells during viral infection. There was no pattern to this IFN production but it occurred on at least one occasion in all animals between 1–14 dpi.

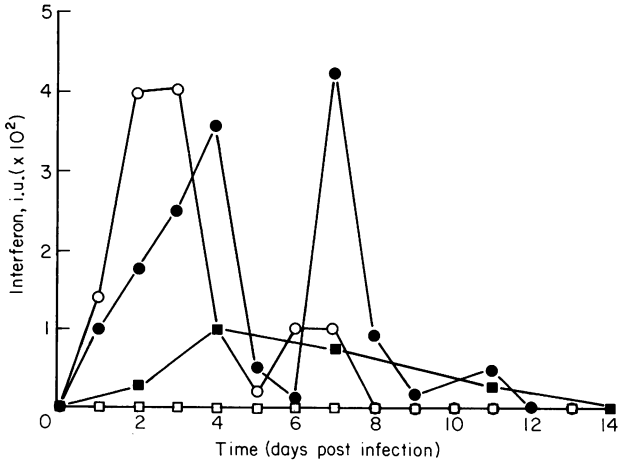

Fig. 5. Interferon levels (i.u.) versus time (days post infection) in EHV-1 subtype 1 (group 1 mares) or subtype 2 (group 3 yearlings) infections. (●, ■) Subtype 1 infection; (○, □) indicate subtype 2 infection; (●, ○) nasal IFN; (■, □) serum IFN.

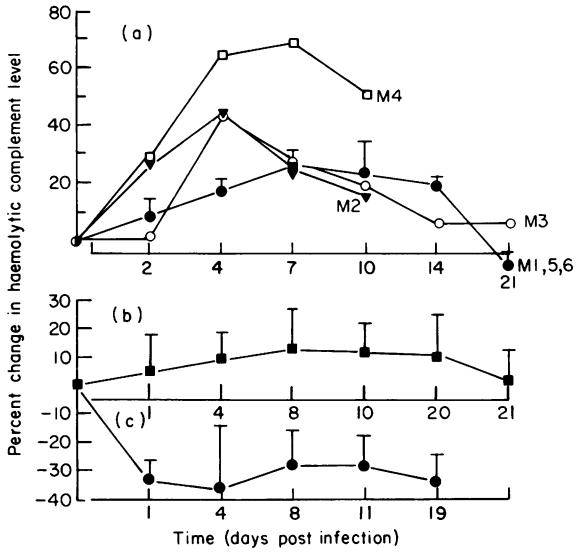

Fig. 6. Per cent changes in equine haemolytic complement levels after experimental viral infection. Horse group identity; (a) EHV-1 subtype 1 infected mares (numbered where appropriate individually, M1–M6); (b) EHV-1 subtype 2 infected yearlings and (c) Equine adenovirus infected yearlings.

Haemolytic complement levels

The CH_{50} titres remained within the normal range during the course of all experimental viral infections. However, the per cent change in complement levels gave varying pattern between and within groups (Fig. 6). Adenovirus infection produced a 35% fall in resting values by 4 dpi which gradually improved, but remained subnormal, up to 19 dpi. On the other hand, EHV-1 subtype 2 infected yearlings and unaffected mares in group 1 showed a moderate rise in complement levels. This appeared as a 10–30% rise between 7 and 10 dpi (Fig. 6). EHV-1 subtype 1 infected mares (M2,3,4), which became paretic, developed a more acute and greater rise in complement levels. The maximum rate of change was 17·8% day in the ataxic mares; 4·5% day in the unaffected mares; and 1·3%/day in the subtype 2 infected yearlings. At 21 dpi CH_{50} titres had returned to resting values in horse groups 1 and 3.

DISCUSSION

We have failed to note disorders of cellular or humoral non-specific immunity prior to infection in horses which eventually succumb to EHV-1 induced paresis. There was also no difference in the duration or degree of viraemia between paretic and non-paretic subtype 1 infected horses. In ataxic animals EHV-1 infection produces, as a result of lytic growth or immune attack, sequelae of haemorrhage and thrombosis (Edington *et al.*, 1986). Taken together this suggests that paresis is not necessarily due to an immunological defect of virus containment, but determined by individual susceptibility to virus infection of the CNS vascular endothelium. Nonetheless, it is conceivable that primary defects only become apparent in these animals as a result of encounter with virus.

Whilst intrinsic ingestion by monocytes during EHV-1 subtype 1 infection was not abnormal when compared to the resting normal range, it was clearly reduced with regard to phagocytic activity during uncomplicated virus infections. The rise in monocyte ingestion observed with EHV-1 subtype 2 and adenovirus infections therefore suggests that clinical tests may be misinterpreted if due regard is not given to transient, but normal, alterations in host immune status.

The decrease in intrinsic phagocytosis and killing by monocytes during subtype 1 infection was in striking contrast to their behaviour during subtype 2 or adenovirus infections. Virus replication is the probable cause of this dysfunction since subtype 1 virus was readily recovered from peripheral blood mononuclear cells whereas, in most animals, subtype 2 or adenovirus did not give a demonstrable viraemia. This conclusion is substantiated firstly by the finding that subtype 1 replicates principally within mononuclear cell populations of peripheral blood (Scott, Dutta & Myrup, 1983) and secondly, by our own unpublished flow cytometer analysis which indicates that 20–25% of circulating monocytes are infected during subtype 1 infection. It seems unlikely that immune complexes *per se* were responsible for a decrease in phagocytosis as PMNs were unaffected. Deterioration of intrinsic MNP function and concomitant immunosuppression are sequelae of virus replication (Rice, Schrier & Oldstone, 1984). However, the extent of immunosuppression in EHV-1 subtype 1 infected horses remains to be determined (Dutta, Myrup & Bumgardner, 1980).

Examples of virus mediated PMN dysfunction have been described (Larson & Blades 1976; Abramson *et al.*, 1982). However, EHV-1 subtype 1 selectively inhibits monocyte function leaving PMNs unscathed. Adenovirus infection, like EHV-1 subtype 1, produced an increase in killing efficiency but was offset by a sharp drop which cannot be explained by viraemia.

The reason for the enhanced functions of PMN killing and monocyte engulfment of *C. albicans* by cells from infected ponies has not been established in this study but might stem from the immunoregulatory activities of interferon (Rhodes, Jones & Bleehen 1983; Einhorn & Jarstrand, 1984: Shalaby *et al.*, 1985; Babiuk *et al.*, 1985). Locally produced IFN was evident in nasal secretions of both EHV-1 subtype 1 and subtype 2 infected ponies. Moreover, IFN production by mononuclear cells was normal, even during subtype 1 infection, demonstrating that this property is maintained despite other infection associated immune dysfunction.

Complement levels may provide an immediate assessment of disease severity in horses with EHV-1 subtype 1 infection since animals which subsequently developed paresis also displayed evidence of increased complement synthesis without consumption. Monocytes respond to inflammatory stimuli by increasing their secretion of complement components (Whaley, 1980) which are available for regulation of immune complex size (Naama *et al.*, 1983; 1985). Immune complexes form during EHV-1 subtype 1 infection but not subtype 2 (Edington *et al.*, 1986) which implies that the immune complexes produced in paretic horses are poor activators of complement or that the complement system itself is faulty. We have postulated (Edington *et al.*, 1986) that subneutralizing subtype 2 antibody may predispose animals to severe infection by subtype 1 virus in a manner analogous to the 'original antigenic sin' model for Dengue Haemorrhagic Fever (Halstead, 1970). It is possible that complexes formed with inappropriate, low affinity anti-subtype 2 antibody and subtype 1 virus not only enhance infection of monocytes (Halstead & O'Rourke, 1977) but are also relatively poor activators of complement; (preliminary results from our laboratory indicate that enhancing antibody is present in subtype 2 exposed animals). Moreover,

ineffective complement activation and reduced C3 deposition on virus particles may also prevent efficient activation of phagocyte viricidal properties (Bridges & Valdimarsson, 1985).

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