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IMMUNOSUPPRESSIVE EFFECTS INDUCED BY CYCLOPHOSPHAMIDE AND CYCLOSPORINE A IN MICE INFECTED WITH EQUINE HERPESVIRUS TYPE-1 (EHV-1)

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SUMMARY

Mice inoculated intranasally with EHV-1 exhibited mild clinical signs of the disease. Severe clinical signs and high mortality (75-100%) were observed when mice were treated everyday with cyclosphosphamide (200 mg/kg/day) or cyclosporine A (40 mg/kg/day) and primarily inoculated with 10^6 PFU. EHV-1. Low IgG antibody titres were detected in the mice treated with cyclosporine A but undetected with cyclophosphamide. In either case, virus titres in the respiratory tissues were significantly increased and correlated with the level of viraemia. Prolonged viraemia was noted in the mice treated with cyclosporine A. The mice that were previously immunised with EHV-1 antigens appeared to be more resistant to cyclosporine A than cyclophosphamide. Upon treatment and challenge virus inoculation, clinical signs with an increase in virus replication were observed but not the mortality. No amnestic IgG antibody response was observed in the mice which were treated with cyclophosphamide. In contrast, the antibody titres in cyclosporine A-treated mice were increased but to lower than those in untreated immunised-mice.

Keywords: EHV-1, immunosuppression, cyclophosphamide, cyclosporine A, virus replication, IgG antibody

INTRODUCTION

The importance of the disease caused by equine herpesvirus type-1 (EHV-1) in horses has been discussed elsewhere (Bryans and Allen, 1989; Azmi, 1995). In general, infected horses displayed respiratory problems, abortion in mares and sometimes paralysis. The disease is widespread and probably most horses in the field already harbor the virus. Horses that recover from the clinical disease may subsequently harbor latent virus, and trigeminal ganglia were suggested to be the latency site (Slater *et al.*, 1994). The latent virus can be re-activated naturally upon stress stimuli or experimentally with corticosteroids (Edington *et al.*, 1985). The use of corticosteroids, however, did not always result in re-activation. Re-activation may occur and be followed by the development of clinical disease irrespective of the presence of neutralising antibody (Bryan and Allen, 1986). However, none of the previous studies described the relationship between stress, immunosuppression, infection and immune responses against EHV-1. Such studies could perhaps be carried out by use of certain immunosuppressive drugs that are capable of suppressing specific immune components. The purpose of the present study in mice infected with EHV-1 is to assess the effects of immunosuppression on virus replication, viraemia, antibody responses and protection by cyclo-

MATERIALS AND METHODS

Virus and cell culture

EHV-1 strain AB 4 and its pathogenicity in horses and mice have been well characterised (Awan *et al.*, 1990; Gibson *et al.*, 1992). The virus was grown in rabbit kidney (RK-13) cells in Earle's minimum essential medium (EMEM) supplemented with 8% foetal calf serum.

Cyclosporine A and cyclophosphamide

Cyclosporine A (Sandimum) and cyclophosphamide (Endoxana) were obtained from Sandoz Pharmaceuticals and ASTA Medica Ltd, UK respectively.

Experiment 1

The effect of cyclophosphamide and cyclosporine A upon primary virus inoculation was studied. Three groups of twenty female BALB/c mice (four week old) were used in this experiment. Starting from 2 days prior to virus inoculation, each group of mice was injected everyday subcutaneously (s.c.) with either cyclophosphamide (200 mg/kg/day), cyclosporine A (40 mg/kg/day) or drugs diluent (served as controls), phosphate-buffered saline (PBS). Two days later, all groups of mice were inoculated intranasally (i.n.) with EHV-1. An inoculum of 40 μL containing 10^6 plaque

nostrils and allowed to be inspired. The inoculation was done under light general anaesthesia. The drug treatments in surviving mice were continued for 28 days p.i. Blood (in EDTA) were collected at days 3, 5, 8 and 14 post-infection (p.i), and subjected for virus infectious centre assay. Serum samples were obtained at days 0, 3, 5, 8, 14, 21 and 28 p.i. and subjected for ELISA test to determine IgG antibody titres. Four mice were killed at days 3, 5, and 8 p.i. and virus titres in the respiratory tissues (nasal turbinates and lungs) were determined.

Three groups of sixteen mice were also given similar treatments as above and inoculated with the same amount of EHV-1. Other two groups of eight mice (uninfected controls) were each treated with either cyclophosphamide or cyclosporine A and uninfected. Clinical signs were observed everyday and cumulative mortality in each groups of mice was determined at days 3, 5, 8, 10, 14 and 21 p.i. The mortality was used as a parameter to measure the severity of the disease.

Experiment 2

The effect of cyclophosphamide and cyclosporine A upon secondary virus inoculation was studied. Three groups of 20 mice were given a primary i.n. inoculation with 2 x 10^6 PFU EHV-1. Four weeks later, two groups of these mice were treated either with cyclophosphamide or cyclosporine A at the same doses as mentioned in experiment 1. The third group was injected with PBS only and served as controls. Two days later, all mice were given a second i.n. inoculation with 5 x 10^6 PFU EHV-1. The drug treatments were then continued for 24 days p.i.

Blood (in EDTA) was collected at days 3, 5 and 8 post-infection (p.i), and subjected for virus infectious centre assay. Serum samples were obtained at days 0, 3, 5, 8, 14, 19 and 24 p.i. and subjected for ELISA test to determine IgG antibody titres. Four mice were killed at days 3, 5, and 8 p.i. and virus titres in nasal turbinates and lungs were determined.

Virus isolation and plaque-forming assay

Mice were killed at days 3, 5 and 8 p.i. The lungs and nasal turbinates were collected separately, minced, homogenised and further disintegrated in a sonic water-bath and centrifuged at 3,000 rpm for 10 min. The supernatants were assayed for virus in RK 13 cells in 24-well cell culture plates and overlayed with 1% carboxylmethylcellulose. After 2 days of incubation at 37°C, plaques were stained with crystal violet solution and counted.

Infectious centre assay

An infectious centre assay was used to assess cell associated viraemia. The blood was collected in the presence of EDTA (2 mg/mL) and centrifuged at 3,000 rpm for 10 min. The buffy coat was separated and treated with distilled water to lyse the erythrocytes. The number of cells was counted in a Neubauer RK-13 cells for 8-10 days. The number of plaques developed was determined and expressed per 10^6 buffy coat cells.

ELISA

An indirect ELISA was developed based on well established principles and protocols (Voller *et al.*, 1980); this has been described previously (Azmi, 1995). 0.5 mg viral antigen was coated onto each well of 96-well plate overnight prior to the addition of blocking agent and serum samples. Serum samples were previously serially diluted two fold, beginning from 1:100 dilution. Goat-anti mouse IgG peroxidase-labelled conjugate was added and allowed to react with the substrate 2,2' azino bis(3 ethylbenzthioline 6 sulfonic acid). The result was read at a wavelength of 492 nm and end point titres determined. Hyperimmune sera with the titre of 1:100,000 and preimmune sera were included as positive and negative controls, respectively.

Statistical analysis

The statistical significance of differences of virus and antibody titres between groups of data was determined using the two tailed Student's unpaired t test.

RESULTS

The effect of immunosuppression on primary virus infection

Following drug treatments and virus inoculation of the first experiment, mild clinical signs including ruffled hair and tachypnoea were noted in the control mice with approximately 19% mortality (Table 1). In general, the mice treated with cyclosphosphamide developed the most severe disease. A mortality of 75-100% was observed by days 5-10 p.i. A less severe disease was observed in the mice treated with cyclosporine A with cumulative mortality of approximately 40-75% by days 5-10 p.i. No death was observed in both groups of mice that were treated with either drugs but uninfected.

Virus titres in the nasal turbinates and lungs of control mice decreased from 4.0-4.5 $\log_{10}$ at day 3 p.i. to 2-3 $\log_{10}$ at day 5 p.i. and undetectable by day 8 p.i. ($P<0.05$) (Figure 1a and 1b). In contrast, significantly high ($P<0.01$) virus titres were noted in the cyclo-phosphamide-treated mice at days 5 (approximately 5 $\log_{10}$ PFU) and 8 p.i. (5.6 $\log_{10}$ PFU). Virus titres could not be determined further since all mice of this group died by day 10 p.i. An increase of approximately 5 $\log_{10}$ PFU at day 8 p.i. was noted in the mice treated with cyclosporine A. However, by day 14, virus titres in the nasal turbinates and lungs of surviving mice treated with cyclosporine A decreased to < 1.5 $\log_{10}$ (data not shown). At any point in time tested, there was no significant difference in virus titres of the nasal

Table 1. Cumulative mortality of mice immunosuppressed either with cyclophosphamide or cyclosporine A following inoculation with 10^6 PFU EHV-1.

	Inoculation	Mortality (days p.i.)					
Drug	EHV-1	3	5	8	10	14	21
PBS	+	0/16	3/16	3/16	3/16	3/16	3/16
Cyclophosphamide	-	0/8	0/8	0/8	0/8	0/8	0/8
Cyclophosphamide	+	8/16	12/16	15/16	16/16	16/16	16/16
Cyclosporine A	-	0/8	0/8	0/8	0/8	0/8	0/8
Cyclosporine A	+	2/16	7/16	10/16	12/16	12/16	12/16

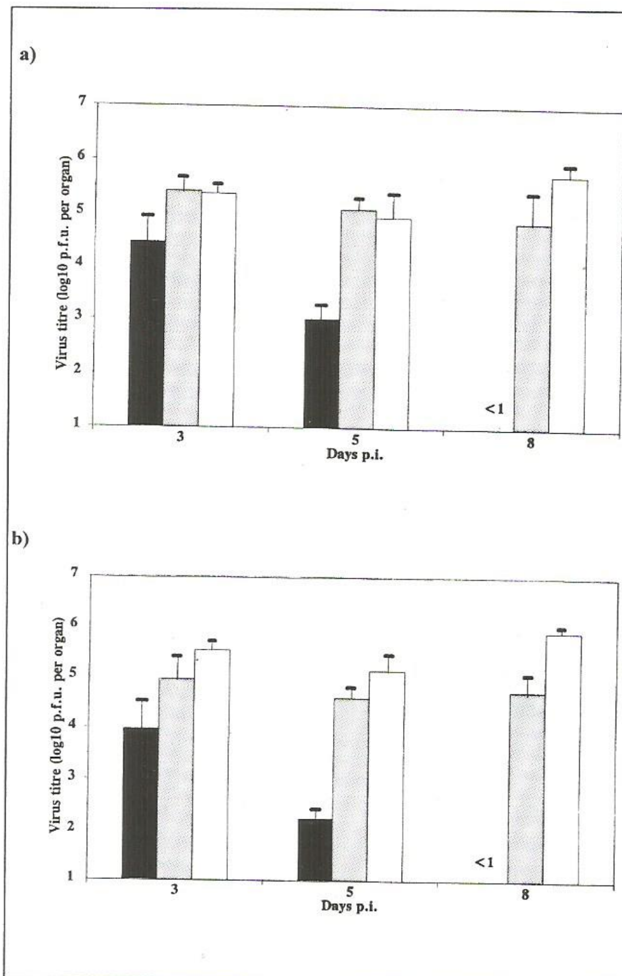

Figure 1. Virus titres (geometric mean; n=4) in the (a) nasal turbinates, and (b) lungs of mice that were treated with cyclosporine A (❐) cyclophosphamide (❐), or PBS (■) and primarily inoculated with EHV-1.

Following the virus inoculation, viraemia was detected in all groups of mice. The level of viraemia was the least in the control group and gradually disappeared by days 8-14 p.i. (Figure 2a). The highest level viraemia detected was in the mice treated with cyclophosphamide, which increased to a maximum by day 8 p.i. (Figure 2b). The level of viraemia in this group of mice could not be determined beyond 10 days p.i. due to 100% mortality. A low level of viraemia was detected in the mice treated with cyclosporine A (Figure 2c). The level of viraemia increased by day 5 p.i. then decreased by day 8 p.i. and was detected at low level by day 14 p.i.

Control mice produced IgG antibody with a peak titre of approximately 0.75 $\log_{10}$ by day 14 p.i. (Figure 3). The mice treated with cyclosporine A produced less IgG antibody with the titre of <0.4 $\log_{10}$. However, at no time IgG antibody was detected in the mice treated with cyclophosphamide.

The effect of immunosuppression on secondary virus infection

Following the drug treatments and second virus inoculation in the second experiment, the mice of the control group displayed mild clinical signs which appeared only on days 1-3 p.i. and fully recovered. Virus titres in the respiratory tissues rapidly decreased from approximately 3 $\log_{10}$ PFU at day 3 p.i. to an undetectable level (<10 PFU) by day 5 p.i. (Figure 4). The virus titre in the nasal turbinates of mice that were treated with cyclosporine A did not increase. However, the virus titre in the lungs increased to approximately 3.85 $\log_{10}$ and 1.3 $\log_{10}$ PFU at days 3 and 5 p.i. respectively, but undetectable at day 8 p.i. The mice which were treated with cyclophosphamide displayed a greater effect on virus titres. An increase of approximately 0.75 $\log_{10}$ PFU (to 3.7 $\log_{10}$ as compared to 2.95 $\log_{10}$ in the control group) was noted in the nasal turbinates at day 3 p.i. The virus titre was however rapidly decreased to 1.2 $\log_{10}$ PFU by day 5 p.i. and undetectable by day 8 p.i. A greater virus increase was noted in the lungs. At any point in time, virus titres in the lungs of mice which were treated with cyclophosphamide were found to remain higher than those in the mice treated with cyclosporine A or to those in the control group. Approximately, an increase of 1.7 $\log_{10}$ PFU was noted in the lungs at day 3 p.i. (with the virus titre of 4.7 $\log_{10}$) but it gradually declined by days 5 (2.2 $\log_{10}$ virus titre) and 8 p.i. (1.75 $\log_{10}$ virus titre). A low level of viraemia (<20 infectious centres per 10^6 buffy coat cells) was also noted in mice of all groups and became undetectable by day 5 p.i. (data not shown).

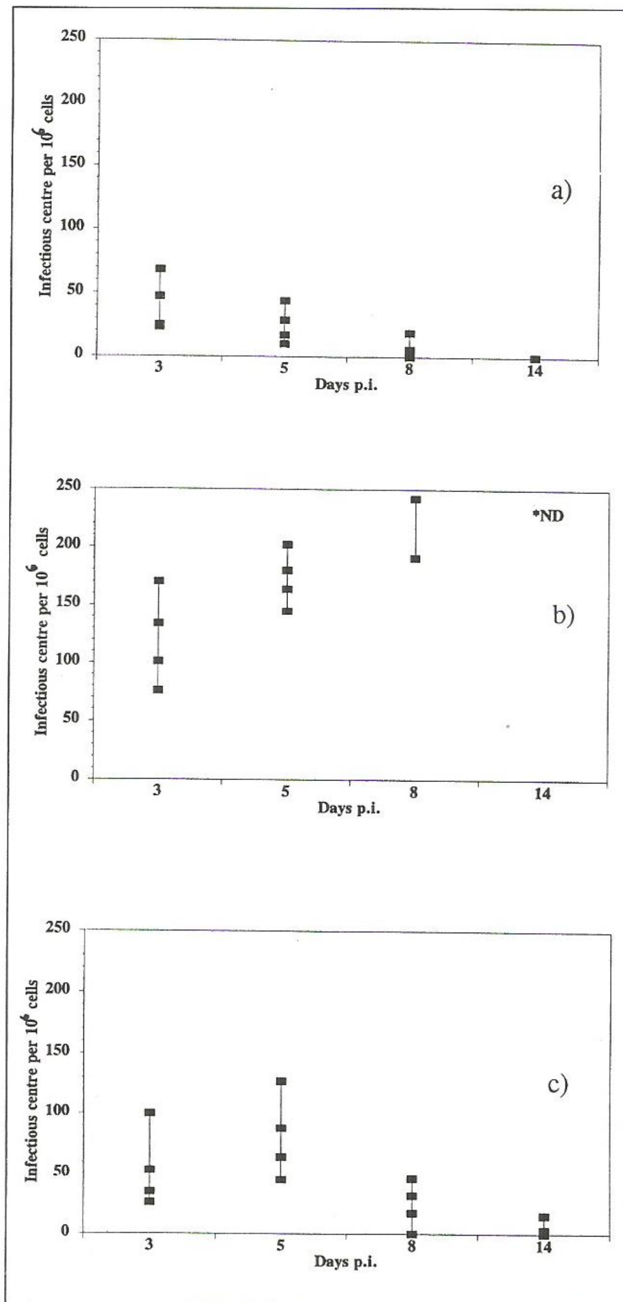

Figure 2. The level of viraemia as represented by the number of infectious centres. The mice were treated with (a) PBS, (b) cyclophosphamide, or (c) cyclosporine A and primarily inoculated with EHV-1. Each data point (■) on the high-low line represented the mean of infectious centres per 10^6 buffy coat cells of individual mice.

Prior to the drug treatments and second virus inoculation, the serum IgG antibody titre detected was approximately 0.5 $\log_{10}$ (Figure 5). Following the virus inoculation, control mice produced IgG antibody of which increased to the titre of 1.7-2.0 $\log_{10}$ by days 5-8 p.i. In contrast, the antibody titres in the mice treated with cyclophosphamide always remained low, <0.75 $\log_{10}$. at all time p.i. Moderate antibody responses were noted in the mice treated with cyclosporine A with the titres of >0.8 $\log_{10}$ beyond day 5 p.i. In general, there was a trend where antibody titres in cyclophosphamide-treated and untreated mice decreasing after reaching their peaks at 8 to 14 days p.i. respectively.

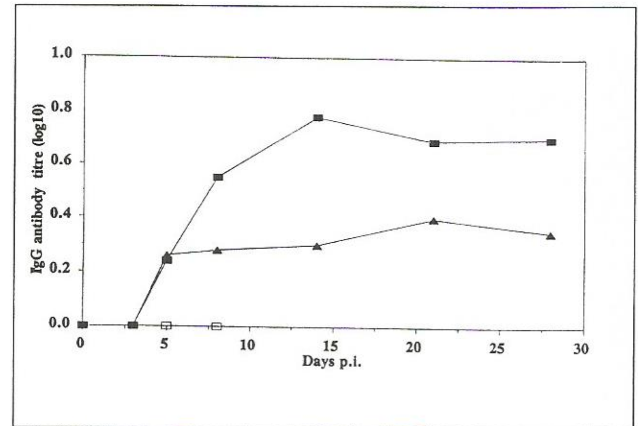

Figure 3. IgG antibody titres (geometric mean; n=4) in the mice which were treated with PBS (■), cyclophosphamide (❒), or cyclosporine A (▲) and primarily inoculated with EHV-1.

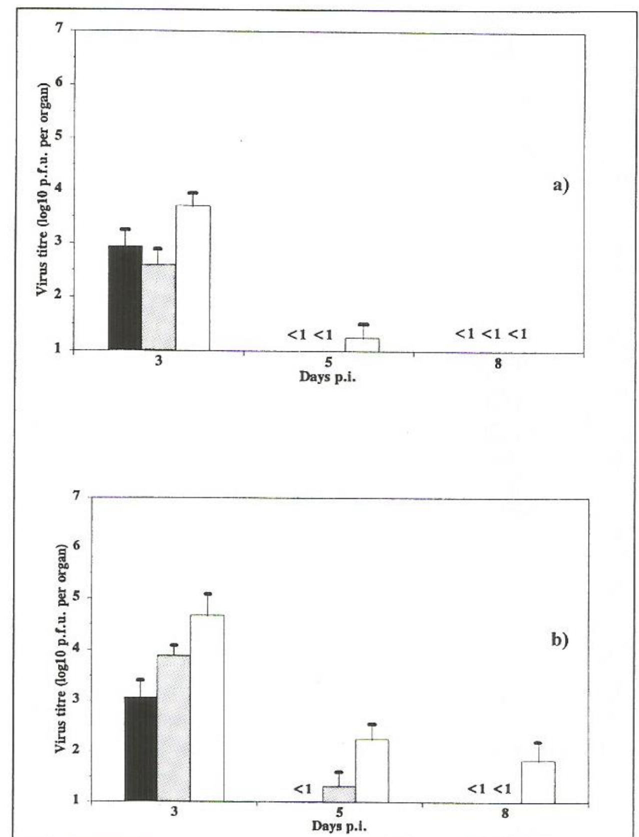

Figure 4. Virus titres (geometric mean; n=4) in the (a) nasal turbinates, and (b) lungs of mice primarily inoculated with EHV-1 antigens. Four weeks later, they were treated with cyclosporine A (❒), cyclophosphamide (❒), or PBS (■) and given challenge inoculation.

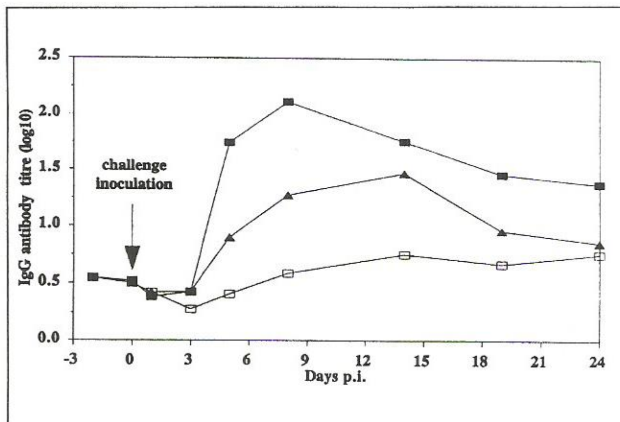

Figure 5. IgG antibody titres (geometric mean; n=4) in the mice as described in Figure 4. The mice were given PBS (■), cyclophosphamide (□), or cyclosporine A (▲).

DISCUSSION

The present study highlighted several important features of EHV-1 infection in mice during immunosuppression induced by immunosuppressive drugs: 1) the treatment with cyclophosphamide resulted in unchecked virus replication and rapid 100% mortality, 2) cyclophosphamide effectively suppressed antibody production, 3) cyclosporine A sustained virus replication and induced prolonged-viraemia without resulting in 100% mortality, 4) cyclosporine A partially blocked antibody production, and 5) protection against second infection was less affected by the treatment with cyclophosphamide or cyclosporine A.

Mice inoculated with 10^6 PFU EHV-1 normally exhibited very mild sign of illness. However, with a similar virus inoculum dose for primary inoculation, the mice that were treated with cyclophosphamide developed severe clinical disease. The 100% mortality is suggested to be related with an extensive damage in the respiratory tissues due to, in part, enhanced virus replication. The production of IgG and perhaps IgM antibody was suppressed to an undetected level. It is suggested that this phenomenon might be directly related to an abrogation of B lymphocytes by cyclophosphamide (Mackie, 1981).

When the mice previously immunised with EHV-1 were given cyclophosphamide and challenge inoculation, IgG antibody responses were suppressed and virus replication in the respiratory tissues was higher than those in cyclopsporine A-treated and untreated mice. However, the gradual disappearance of the virus from the respiratory tissues and blood cells may indicate not all sensitised-immune cells can be suppressed. This may help the clearance of infectious viruses from infected tissues and blood cells. Since B lymphocytes were suppressed, the humoral factor is not suggested to be a critical factor for the clearance of infectious virus, perhaps cell-mediated is of importance.

Following primary virus inoculation, immunosuppression with cyclosporine A resulted in development of clinical disease but in a less severe form than those treated with cyclophosphamide. Some infected-animals may survive despite the presence of high virus titres in respiratory tissues. By contrast, a previous *in vitro* study using cyclosporine A in the cell culture infected with herpes simplex virus type 1 (Vahlne *et al.*, 1992) indicated the occurrence of suppression in virus replication. It is suggested the reduced mitotic activity of the cyclosporine A-treated cells may account for the restricted-virus replication *in vitro*. In the present *in vivo* study, cyclosporine A may directly suppress T cells (Jenkins *et al.*, 1988), and that would allow unchecked virus replication to occur in the respiratory tissues.

The presence of serum IgG antibodies indicated the function of B lymphocytes was not suppressed by cyclosporine A. Since only T_{H1} and not T_{H2} lymphocytes were suppressed by cyclosporine A (Kahan, 1989), antibodies could be produced although at low level. Thus, the presence of IgG antibodies may help to reduce the number of infectious virus but this seemed to occur beyond day 10 following primary infection when the antibody titres reached it peak. Furthermore, the clearance of the virus from the blood was evidenced by day 14 p.i. concomitant with the increase of antibody responses. Surprisingly, IgG antibodies titres in cyclosporine A-treated mice were lower but the virus titres were significantly reduced in a similar trend to those in untreated mice. This indicated that the immune responses against EHV-1 in the previously immunised individuals were not effectively suppressed by cyclosporine A. This may be supported by the previous report which indicated primed-T lymphocytes (but not non-primed T lymphocytes) resistant to cyclosporine A (Kahan, 1989).

In general, virus titre in the lungs is more vulnerable to changes than those in turbinates following treatment with either drug. Perhaps this was due to differences in the distribution and kinetics of the drugs in different tissues (which has yet to be defined). Based on the present study, it is suggested that the lung tissue is more susceptible than that of the nasal turbinate. In the method used to study cell-associated viraemia, the virus can only be seen replicating following 8-10 days co-cultivation and in some cases required second passage in tissue culture. In contrast, only 3 days cultivation was required for the presence of replicating viruses in cell culture using other infected-tissues. Thus, it is suggested that the virus may also remain latent in the blood cells.

In conclusion, at the dose employed, cyclophosphamide is a potent immunosuppressor of antibody responses and it gave the greatest effect, particularly in previously immunised mice, in enhancing virus replication in the lung but was less effective in the nasal turbinate. Cyclosporine A effectively sustained virus replication and prolonged

viraemia during primary infection but was less effective if the animals have been previously immunised. Based on the present study, it is suggested that immunosuppression of antibody responses in horses may also result in enhanced virus replication. Enhanced and prolonged viraemia occurred during immunosuppression could be exploited to study the role of viraemia that may be of importance for the occurrence of latency, reactivation, transplacental infection and infection of the central nervous system in horses.

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RINGKASAN

KESAN IMUNOTEKAN TERARUH SIKLOFOSFAMIDA DAN SIKLOSPORIN A DALAM MENCIT TERJANGKIT DENGAN HERPES VIRUS EKUIN TIP-1 (EHV-1)

Mencit terinokulat intranasum dengan EHV-1 mrnunjukkan petanda klinikal ringan penyakit. Petanda klinikal dan kadar kematian tinggi (75-100%) telah mencerapkan apabila mencit diperlakukan setiap hari dengan siklofosfamida (200 mg/kg/hari) atau siklosporin A (40 mg/kg/hari) dan secara primer diinokulatkan dengan 10^6 PFU *EHV-1. Titer antibodi IgG yang rendah telah dikesan dalam mencit yang diperlakukan dengan siklosporin A tetapi tidak dikesan apabila mengguna siklofosfamida. Dalam kedua-dua kes, titer virus dalam tisu pernafasan adalah tertingkat secara tererti dan terkorelasi dengan aras viremia. Viremia berpanjangan telah dilihat dengan mencit yang diperlaku dengan siklosporin A. Mencit yang terdahulunya diimunkan dengan antigen EHV-1 nampaknya lebih tahan siklosporin A daripada siklofosfamida. Berikutan perlakuan dan penginokulatan virus cabaran, apa yang dilihat ialah petanda klinikal dengan peningkatan pereplikatan virus, dan bukan peningkatan kadar kematian. Tiada gerakbalas antibodi IgG amnestik dicerapkan dalam mencit yang diperlaku dengan siklofosfamida. Disebaliknya, titer antibodi dalam mencit terperlaku siklosporin A meningkat tetapi hanya kepada aras yang lebih rendah daripada yang berlaku dalam mencit terimun bukan terperlaku.*