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A Restrictive Virus Tropism, Latency and Reactivation of Pseudorabies Virus Following Irreversible Deletion of *Bsr*I Restriction Site in the Thymidine-kinase Gene

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At the dose of 1000 p.f.u. per mouse, 100% mortality occurred in mice inoculated with wild-type pseudorabies virus (PrV). In contrast, upon stable deletion of 10 bp nucleotides at the *Bsr*I site within the TK gene, PrV was rendered to be completely apathogenic. The deletion also caused the virus to be less capable of replicating in respiratory as well as in nervous system tissues. Although animals were exposed to high titers of TK-deleted PrVs, the virus failed to replicate to a high titer as compared to the pathogenic parental virus. In contrast to previous studies, the deletion in the TK gene did not prevent the virus from establishing latency. Upon immunosuppression, the latent virus, however, reactivated but replicated at low titers. Interestingly, TK-deleted virus established latency and reactivation, that are occurred only in trigeminal ganglia and the cerebrum, and no other tissues involved. Following reactivation, there was no indication of virus shedding in respiratory tissues as confirmed by virus isolation and polymerase chain reaction (PCR) technique targeting at the gB gene of PrV. The non-pathogenic virus with non-shedding characteristics, upon reactivation of the latent virus, would be the important feature of a live virus vaccine candidate.

Key words: pseudorabies virus, thymidine kinase, latency, reactivation, tropism

Pseudorabies virus (PrV) infects several species of animals including pigs, which are the major natural host and reservoir. Cats, dogs, cows, rabbits and mice are also susceptible to the infection but the pseudorabies disease developed in these species is rather limited (Kluge *et al.*, 1992). Following virus infection, the virus replicates in epithelial cells underlining the mucosa surface of the respiratory tract. Subsequently, a direct virus uptake into nerve axons occurs where the virus is being transported centripetally via retrograde fast axonal transport to cell bodies of neurons in sensory and autonomic nerve ganglia. Similar to other alphaherpesviruses, PrV establishes latency in the host. Most alphaherpesviruses establish latency in neural tissues, mainly within the neurons of ganglia with varying duration (Stevens, 1989; Ahmed and Stevens, 1990). There is a general consensus that neurons harbor the latent virus. Latency and reactivation of latent viruses are significant in the biology of recurrent infection and subsequent virus transmission. The virus also may establish latency in other types of cells or tissues. However, studies conducted previously on latency in non-neuronal tissues are rather limited and not conclusive.

The exact physical state of the virus particle during latency in unknown. Generally, latent viruses are unlikely to replicate or express viral proteins. During the latent state, complete virions and viral immunogenic glycoproteins are not expressed and not readily available to the immune system. This renders the antigen-dependence immunosurveillence system unable to detect the presence of latent viruses in the host. Viruses in the latent state produce a set of RNA transcripts, namely latency-associated transcripts (LATs). Other than LATs, there are no other viral transcripts detectable by known molecular techniques. LATs may play some role in the establishment of latency and viral reactivation. Other than LATs, PrV DNA is also present in neuronal tissues primarily as a linear molecule (Rziha *et al.*, 1986) but not integrated in the host chromosomal DNA. Approximately 20 to 30 copies of this viral genome may be present in individual cells. The low number of DNA copies available may indicate that the original multiplicity of infection required for the establishment of latency in the individual infected cells is minimal.

Periodically, the latent virus can be reactivated spontaneously or upon exposure to noxious stimuli including ultraviolet light, fever and stress. Chemical agents related

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to downregulation of the nervous, endocrine and immune systems have been used experimentally to reactivate latent herpesviruses (Blondeau and Aoki, 1992; Tenser *et al.*, 1993). Synchronous reactivation of PrV could be achieved following a single intravenous dose of glucocorticoides such as dexamethasone (Thawley *et al.*, 1984). Many latent herpesviruses can also be reactivated experimentally upon injection with neurotransmitters such as epinephrine (Martin and Suzuki, 1987; Bloom *et al.*, 1994) and acetylcholine (Tanaka *et al.*, 1996). The molecular process underlining viral reactivation as induced by these stimuli is poorly understood. Probably, it occurs by a direct implication of transactivity of immediate-early (IE) regulatory genes that causes switching from latency to lytic growth.

The mechanisms of latency and reactivation are important from the disease control standpoint. The virus that establishes life-long latent infection in the host could be responsible for perpetuation and transmission of PrV in nature. The ultimate control strategies for PrV must therefore not only prevent clinical diseases but must also interrupt virus transmission in nature. An improved understanding of the establishment, tropism, maintenance and reactivation of latent viruses is important. Such knowledge could facilitate the formulation of novel strategies that would lead to the preparation of appropriate virus vaccines. Such vaccines should be valuable for the prevention of latent infections or prevent reactivation and subsequent virus transmission to other animals.

The purpose of this study was to investigate the effects of irreversible deletion of thymidine-kinase (TK) gene on the virus's capability to replicate, virus tropism, latency and reactivation in mice. Data obtained shall be used to determine the suitability of the TK-gene-deleted virus as a candidate for a pseudorabies vaccine.

Materials And Methods

Virus

A thymidine-kinase (TK) gene-deleted PrV was prepared at Virology Research Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia. The parental wild-type virus was obtained from a pig farm during a pseudorabies outbreak in Malaysia. The TK-gene-deleted PrV was constructed by deleting 10 base-pair of nucleotides at *Bsr*I restriction site to cause early termination of the TK gene (Zeenathul, 1999). The mutant was found to be genetically stable as tested in tissue culture and DNA sequencing after it had been passed through tissue cultures 100 times. The working stock of the virus was prepared in Vero cells. All cells were grown in the standard L-15 medium (Gibco BRL, USA) supplemented with 8% fetal calf serum (FCS) (Gibco BRL, USA).

Drugs

Drugs used to reactivate latent viruses in mice were cyclophosphamide monohydrate (Sigma, USA) at the dose of 200 mg/kg body weight and dexamethasone (Sigma, USA) at the dose of 16 mg/kg body weight. These drugs were injected once daily subcutaneously (s.c.) for a maximum 10 consecutive days.

Experiment I

A preliminary experiment was conducted to demonstrate virus replication in mouse tissues. Two groups of eighteen 4-week-old female BALB/c mice were inoculated intranasally (i.n.) with 1×10^6 p.f.u. of thymidine kinase gene-deleted PrV per mouse and 1×10^2 p.f.u. of wild-type PrV per mouse. No mice survived when the dose was more than 1×10^3 p.f.u. of wild-type PrV per mouse. Four mice were killed at days 2, 5, 8 and 10 following virus inoculation. Tissue samples including nasal turbinate, lung, cerebrum, cerebellum and trigeminal ganglia were obtained and subjected to virus assay.

Experiment II

Six groups (A, B, C, D, E and F) of 4-week-old female BALB/c mice (22 mice per group) were employed. Another group (Group G) of 10 mice served as the negative control group. Mice of groups A,B and C were inoculated intranasally (i.n.) with 1×10^2 p.f.u. of wild-type virus. Mice of groups D,E and F were were inoculated intranasally (i.n.) with 1×10^6 p.f.u. of thymidine kinase gene-deleted virus. Mice of group G were inoculated with PBS. Serum samples were collected from each group of mice at days 5, 10, 15, 20, 25 and 30 following virus inoculation and subjected to antibody assay. One month later, 4 mice from each group (except Group G) were sacrificed and tested for the presence of virus in the respiratory and nervous system tissues. Mice were tested for the presence of replicating virus. The mice of group A and D were given cyclophosphamide and mice of groups B and E received dexamethasone while mice of groups C, F and G were given PBS. The drug regime was continued for another 9 days. Two mice of Group G and four mice from each of other groups were killed at days 2, 5, 8 and 10 following the first day of drug administration. Tissues were examined for viruses by plaque forming assay. Tissue samples were also tested in polymerase chain reaction (PCR) for the presence of viral DNA.

Experiment III

Similar to Experiment II, six groups (A, B, C, D, E and F) of 4-week-old female BALB/c mice (24 mice per group) were employed. Another group (Group G) of 10 mice served as a control group. Mice of groups A,B and C were inoculated intranasally (i.n.) with 1×10^2 p.f.u. of wild-type virus. Mice of groups D,E and F were inoculated intranasally (i.n.) with 1×10^6 p.f.u. of thymidine

kinase gene-deleted virus. Mice of group G were inoculated with PBS. Serum samples were collected from each group of mice at one-month intervals following virus inoculation and subjected to antibody assay. At months 1, 2, 4, 6 and 8 following virus inoculation, four mice of group A and D were selected randomly, kept separately and given cyclophosphamide. The similar treatment was applied to mice of groups B and E. The two groups of mice were injected with dexamethasone while mice of groups C and F received PBS. Two mice of Group G were selected randomly and injected with PBS. The drug regime was continued for another seven consecutive days. At day 8 following the first drug administration, mice were killed and their tissues were collected for further tests.

Virus isolation

Mice were sacrificed and nasal turbinate, lung, cerebrum, cerebellum and trigeminal ganglia were collected in 1 ml ice-cold serum-free L-15 medium containing 8% antibiotic-antimycotic solution. Tissue samples were minced thoroughly with scissors and homogenized with an electric homogenizer (Thyristor Regler, Germany). The cell suspension was subjected to sonic vibration for one minute in an ice-cold sonic water bath. The sonication caused the release of cell-associated viruses. The suspension was then transferred to eppendorf tubes and spun at 3,000 rpm for ten minutes at 4°C in a refrigerated centrifuge (Hettich, Germany). The cell debris was discarded and the supernatant was kept at 70°C. Virus detection in supernatants and titration by plaque forming assay were conducted in Vero cell culture.

Virus titration by plaque forming assay

Confluent Vero cell cultures for virus titration were prepared in 24-well tissue culture plates (Costar, USA). Ten-fold serial dilutions of virus samples were prepared in serum-free L-15 medium. One hundred µl of each dilution was inoculated in duplicates onto the confluent monolayers. The virus was allowed to adsorb to cells at 37°C for 45 min. Five hundred µl of L-15 overlay medium containing 2% FCS, 1.2% CMC, 1% antibiotic-antimycotic and 1% anti-PPLO solutions was added to each well. The plate was incubated at 37°C. After 3 to 4 days incubation, the overlay medium was discarded and the monolayer was rinsed twice gently in PBS. The cell monolayer was fixed for 10 min in 70% methanol and stained in 2.5% crystal violet solution for 5-10 min. The plate was rinsed gently in tap water until the excess stain disappeared. The plate was dried at room temperature and plaques were counted by using an inverted-light microscope (Olympus, Japan). Virus titers were calculated as the mean number of plaques in the last sample dilution that produced plaques multiplied by the reciprocal of that dilution. Titers were expressed in $\log_{10}$ plaque forming unit (p.f.u.) per sample.

Polymerase chain reaction (PCR)

The DNA of PrV obtained from mouse tissues was extracted by the use of a standard protocol as described previously by Mohd-Azmi *et al* (1999). The DNA sample was used in PCR as a template to amplify and detect the 778 bp nucleotides encoding a portion of the glycoprotein B (gB) gene of PrV. The DNA sequences of primers employed in PCR were 5'-ATCTTGTGCAGAACTC-CATG-3' and 5'TCATTGTACCGGATCATGTC-3' (Mohd-Azmi *et al.*, 1999). Fifty µl PCR reaction mixture was prepared in 50 µl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 µM of each dNTP, 1.0 µM of each primer and 2 units of *Taq* polymerase. Prior to thermal cycling, reaction mixtures were heated at 95°C for 4 min. For each cycle, samples were denatured at 95°C for 1 min, annealed at 55°C for 1 min and extended at 72°C for 1 min. The amplification was performed for 30 cycles in a Perkin Elmer-Cetus DNA Thermal Cycler. Upon final extension, the heating period at 72°C was extended for 8 minutes. Samples of 20 µl volume containing the amplified PrV-DNA were subjected to 1% agarose gel electrophoresis at 90 volts for 2 hours. Gels were stained in ethidium bromide and the 778 bp PrV-gB DNA fragment was visualized in UV light transilluminator.

Enzyme-linked immunosorbent assay for detection of serum antibody

An indirect enzyme-linked immunosorbent assay (ELISA) technique was employed with some modifications (Azmi and Field, 1993). The test was carried out with a working volume of 50 µl of each reagent. The PrV antigen was prepared at 10 µg viral protein per ml in bicarbonate buffer. Each well of a 96-well plate (Dynatech Immulon, USA) was coated with the antigen and incubated at 4°C overnight. The plate was washed thee times in phosphate-buffered saline Tween 20 (PBST) by using an automated microplate washer (Dynatech, USA). Bovine serum albumin (2%) was added and incubated at 45°C for two hours to block the non-specific binding. The plate was washed three times as described above. Ten-fold serially diluted test sera were added and the plate was incubated at 37°C for one hour. The plate was washed three times. Fifty µl of pre-diluted goat anti-mouse peroxidase conjugated immunoglobulin (Sigma, UK) was added and allowed to react with the antigen-bound mouse antibodies by incubating the plate at 37°C for one hour. The plate was washed three times. The substrate solution, 2,2-Azino-bis (3-ethylbenzthioline-6-sulfonic acid) (ABTS) (Sigma, UK) prepared in citrate-phosphate buffer (CPB) and supplemented with 0.01% of H_2O_2 was added and the plate was incubated at room temperature for 30 minutes. The plate was read immediately in a spectrophotometer (Dynat-

ech MR7000, USA) at dual wavelength mode at absorbance 410-490 nm. Hyperimmune and preimmune sera were included in the plate as positive and negative controls respectively. End-point titers of serum antibodies were determined by plotting the data, serum dilution against optical density (O.D). The antibody titer is expressed as the $\log_{10}$ dilution of the serum that corresponds to an optical density value ≥ the mean of the wells of preimmune sera plus three times the standard deviation.

Statistical analysis

The statistical significance of differences between groups of data was determined by using the two-tailed Students unpaired t-test.

Results and Discussion

This study has pointed out several important findings in regards with the capability of PrV to establish latency, tropism or predilection sites and reactivation in relation to the role of PrV TK gene. Evidence gathered has shown that other than virus pathogenicity, TK gene is also related with the virus potential to transmigrate between its predilected tissues and affecting virus replicability, latency and virus reactivation.

In the study, mice inoculated with wild-type PrV at a relatively low dose (100 p.f.u. per mouse) developed a severe disease. Mortality observed was 100% when the inoculum dose was beyond 100 p.f.u. per mouse. Notwithstanding higher doses (10^6 p.f.u.) of virus inoculation, mice inoculated with PrV with deletion in the TK gene did not develop any signs of disease. This result indicates that TK-gene is one of PrV's virulent factors (Dean and Cheung, 1995; Boldogkoi *et al.*, 2000). The significantly higher virus titers obtained from particular tissues, especially the cerebrum, indicates the tissues involved were predilection sites of virus replication. As consistently observed throughout the experiments, the highest virus titers detected were in the cerebrum and cerebellum (Table 1). Interestingly, virus titers in the cerebrum and trigeminal ganglia reached a peak on day 5 p.i. and declined significantly to undetectable levels by day 8 p.i. The virus disappearance from trigeminal ganglia coincides with the disappearance of the virus from the cerebellum. The abrupt upset in the increasing trend of virus titers may indicate the virus "ceased" to replicate but this was not evidenced for viruses in the cerebrum. In contrast, viruses in the cerebrum replicated to highest titers on day 5 p.i. then declined, but were detected until day 8 p.i. This suggested that viruses produced in the cerebrum and cerebellum act as the source of infection to trigeminal ganglia after which the virus may then establish latency. Another point, which is of interest, is the replicating virus in the cerebrum may act as an important source of infection to the cerebellum during primary infection. Low virus titers in the lung may indicate low virus preference for its replication in this tissue. The moderate virus titers as determined in nasal turbinate indicates there was a temporary virus replication in nasal mucosa cells following intranasal virus inoculation. Viruses produced therein act as a source of infection to the nerves innervating the tissue. The brief presence of the virus in the lung, following infection either with wild-type or TK-gene-deleted PrV, indicates the pulmonary involvement is not a significant characteristic of pseudorabies disease. Therefore, mortality occurred following PrV infections was unlikely to be due to respiratory disease.

The resulting mortality occurred, following wild-type virus inoculation at as low as 100 p.f.u PrV per mouse, as a result of direct neural involvement. This is evidenced since virus replication in all nervous tissues examined associated with high virus load as shown in most experiments. A different phenomenon was observed in mice inoculated with TK-gene-deleted PrV at much higher inoculation doses, up to 10,000 fold more than that of wild-type virus. Such mutants are normally characterized by reduced virulence and the virus can only be isolated sporadically in infected animals (Vilnis *et al.*, 1998). Interestingly, deletion in the TK gene renders the virus less of capable of replicating in predilected tissues. Deletion in the TK gene had significantly reduced virus capa-

Table 1. Replication of TK-gene-deleted and wild-type PrVs in mice following intranasal inoculation

Virus	Day p.i.	Virus titer				
		Nasal turbinate	Lung	Cerebrum	Cerebellum	Trigeminal ganglia
TK gene-deleted	2	1.39± 0.32	0.95± 1.07	1.05± 0.15	0.33± 0.85	1.37± 1.50
	5	0.87± 0.37	-	1.33± 1.38	0.86± 1.21	1.25± 0.37
	8	-*	-	0.52± 0.85	-	-
	10	-	-	-	-	-
Wild-type	2	2.43± 0.36	0.85± 0.49	2.95± 0.70	2.47± 0.15	1.42± 0.33
	5	0.71± 0.15	0.58± 0.91	2.65± 0.43	2.06± 0.98	0.85± 1.07
	8	-	-	1.03± 0.51	-	-
	10	-	-	-	-	-

*virus titer below the detection level (<10 p.f.u. per organ)

bility to replicate in high titers in respiratory system tissues as well as in nervous tissues. This would reflect the reduction in migration of the mutants in the nervous system as compared with that of the wild-type strain (Ferrari *et al.*, 2000). Despite an extremely high inoculation dose, reduced virus capability to replicate was observed in nasal turbinates, the cerebrum and cerebellum. The high virus titers could be detected in many tissues following inoculation with extremely high virus inoculation doses, but there was no association with development of signs of pseudorabies disease. Interestingly, the capability of this virus to replicate in cell culture was not altered and, in fact, the growth characteristic was similar to that of wild-type PrV (Jin and Scherba, 1999). This evidence showed that a factor or factors are required for the virus to replicate in an efficient manner to sustain virus titers *in vivo*. Hypothetically, these factors are deemed to be supplied by the host and they must interact with the TK gene product. However, such host factors are not required for the virus to replicate *in vitro* as indicated by a normal virus growth in cell culture. The sustainable virus titers in the cerebrum could be supported by retrograde virus transmission via nerves to the nerve endings supplying respiratory tissues and followed by *in situ* virus replication.

Table 2. Virus replication following reactivation of wild-type PrV at day 30 post-virus inoculation

Group	Drug treatment	Day post-treatment	Virus titer				
			Nasal turbinate	Lung	Cerebrum	Cerebellum	Trigeminal ganglia
A	CPS†	0	-*	-	-	-	-
		2	-	-	-	-	0.82± 0.32
		5	-	-	1.09± 1.21	-	1.62± 0.1.5
		8	0.61± 1.31	-	1.62± 0.46	0.48± 1.15	2.13± 1.07
		10	0.82± 1.81	-	1.85± 0.86	-	1.95± 0.33
B	DXM	0	-	-	-	-	-
		2	-	-	-	-	-
		5	-	-	-	-	1.15± 0.15
		8	0.82± 0.86	-	1.39± 1.31	-	1.54± 0.68
		10	-	-	-	-	1.50± 1.18
C	PBS	0	-	-	-	-	-
		2	-	-	-	-	-
		5	-	-	-	-	-
		8	-	-	-	-	-
		10	-	-	-	-	-

*virus titer below the detection level (<10 p.f.u. per organ)
†CPS-cyclophosphamide, DXM-dexamethasone, PBS-phosphate buffered saline

Table 3. Virus replication following reactivation of TK-gene-deleted PrV at day 30 post virus inoculation

Group	Drug treatment	Day post-treatment	Virus titer				
			Nasal turbinate	Lung	Cerebrum	Cerebellum	Trigeminal ganglia
D	CPS†	0	-*	-	-	-	-
		2	-	-	-	-	0.78± 0.49*
		5	-	-	0.86± 0.33	-	1.22± 1.07
		8	-	-	1.15± 0.38	-	1.31± 0.66
		10	-	-	0.93± 1.02	-	1.17± 0.91
E	DXM	0	-	-	-	-	-
		2	-	-	-	-	-
		5	-	-	-	-	0.71± 0.53
		8	-	-	0.98± 0.57	-	1.08± 0.98
		10	-	-	-	-	1.02± 0.24
F	PBS	0	-	-	-	-	-
		2	-	-	-	-	-
		5	-	-	-	-	-
		8	-	-	-	-	-
		10	-	-	-	-	-

*virus titer below the detection level (<10 p.f.u. per organ)
†CPS-cyclophosphamide, DXM- dexamethasone, PBS-phosphate buffered saline

Table 2 and Table 3 show wild-type and TK-gene-deleted PrVs capable of establishing latency that was reactivated upon treatment with immunosuppressive agents. However, characteristics of virus reactivation in one virus were different from the other virus. For both viruses, cyclophosphamide is a better immunosuppressant than dexamethasone in inducing virus reactivation. As indicated by virus titers presented in Table 2, virus reactivation following treatment with cyclophosphamide was characterized by a sustaining virus replication beginning with reactivation in trigeminal ganglia. The virus was then detected in the cerebrum followed by cerebellum and finally in nasal turbinate but not in the lung. Virus titers in trigeminal ganglia remained high and correlated with that in the cerebrum throughout the treatment regime. Virus reactivation following treatment with dexamethasone also was characterized by virus detection initially in trigeminal ganglia followed by the cerebrum and in nasal turbinate. However, virus replication in trigeminal ganglia could not be associated with virus replication in the cerebrum and nasal turbinate. Furthermore, no virus was detected in the lung and cerebellum. Thus, treatment with dexamethasone and virus replication associated with it was of short-term basis and failed to support virus replication in the cerebrum as well as in nasal turbinate. Obviously, this evidence was not involved in the possible spontaneous virus reactivation as there was no virus detected in control animals. The present data also did not support the previous finding where TK-deficient viruses could not be reactivated from the latent state by corticosteroid treatment (Ferrari *et al.*, 2000). Our evidence explicitly shows that TK-deficient viruses have a similar capability to establish latency and reactivation. The difference in the virus capability to reactive has yet to be studied further.

Animals inoculated with TK-deleted-PrV also harbored latent viruses. As indicated in Table 3, reactivated viruses could be detected in trigeminal ganglia and the cerebrum. Consistent with previous results of the wild-type virus, virus reactivation following treatment with dexamethasone was of short duration and was not as successful as that of following treatment with cyclophosphamide. Other than trigeminal ganglia and cerebrum, no virus could be detected in nerve and respiratory tissues supporting the evidence of no virus being shed (Ferrari *et al.*, 1998).

For both types of PrVs, sustaining virus reactivation occurred earliest in trigeminal ganglia. This confirms that a trigeminal ganglion is the site for the virus to establish and remain latent until reactivation upon appropriate stimuli (Smith and Cheung, 1998). Reactivated viruses can be transmitted from the brain to the mucosa of respiratory tissues via nerves supplying the latter, especially via the olfactory bulb (Babic *et al.*, 1994). The virus may replicate therein and shed in the respiratory secretions. Therefore, when the virus is detected in the respiratory tissue, it indicates the virus has been shed from the host, which serves as the source of infection to other animals. Interestingly, there was no virus detected in respiratory tissues of animals following reactivation of TK-gene-delete PrV. Therefore deletion in the TK gene renders the virus unable to shed its progeny upon reactivation of the latent virus. Low virus titers detected following reactivation indicated (i) only a small amount of latent virus available in nervous tissues, (ii) only a small amount of latent virus can be reactivated, or (iii) reactivated latent virus was not capable of replicating to a high titer.

The main sites for the virus to establish latency, in particular, were trigeminal ganglia and the cerebrum. Failure to isolate the virus, upon reactivation regimes, from nasal tissues and the cerebellum may indicate the virus did not establish latency in cells of these tissues. In contrast, the virus was consistently detected in trigeminal ganglia regardless of the presence of the TK gene in the virus. This confirmed that the TK gene is not a determining factor in establishing latency for PrV (Jin and Scherba, 1999).

The PCR technique employed was aimed at detecting the presence of a small amount of the virus to the level

Table 4. Detection of PrV DNA in mouse tissues by PCR following virus reactivation by cyclophosphamide at day 30 post virus inoculation

Group	Virus	Day post-treatment	Detection by PCR				
			Nasal turbinate	Lung	Cerebrum	Cerebellum	Trigeminal ganglia
A	Wild-type	0	-*	-	-	-	-
		2	-	-	4/4	-	4/4
		5	1/4	-	4/4	-	4/4
		8	4/4	-	4/4	3/4	4/4
		10	4/4	1/4	4/4	2/4	4/4
D	TK-gene deleted	0	-	-	-	-	-
		2	-	-	2/4	-	4/4
		5	-	-	4/4	-	4/4
		8	-	-	4/4	-	4/4
		10	-	-	4/4	-	4/4

*undetected

where the conventional virus isolation methods fail to reveal a positive result especially at the early stage of virus reactivation. The target gene of interest for DNA amplification was PrV glycoprotein B (gB) gene (Mohd-Azmi *et al.*, 1999). Results from studies using this technique (Table 4) were consistent with our studies on virus isolation from affected tissues. Generally, animals inoculated with wild-type PrV were tested positive for all tissues tested. Variation in results was obtained especially for the cerebrum and lung samples at different time p.i. This may reflect the virus spread among predilected tissues limited by certain host factors. In contrast, PCR technique applied to respiratory tissues of animals inoculated with TK-deleted-PrV could not detect viral DNA. This result was in concordance with the findings of a previous study (Ferrari *et al.*, 1988). Viral DNA detected in nervous tissue was only in the cerebrum and cerebellum, consistent with results of virus isolation. Subsequently no virus, following reactivation and replication, was transported from trigeminal ganglia and cerebrum to other tissues. These results also indicated the virus was unlikely to remain latent in tissues of non-nervous origin. This study confirmed that trigeminal ganglia and the cerebrum are important sites for virus latency and reactivation independent from the function of the TK gene (Smith and Cheung, 1998). However, the TK gene is required for active

Table 5. Virus reactivation (at day 8 post-treatment) in mouse tissues for a period of eight months after a single exposure to wild-type PrVs

Group	Drug treatment	Month p.i.	Virus titer				
			Nasal turbinate	Lung	Cerebrum	Cerebellum	Trigeminal ganglia
A	CPS†	1	0.71± 1.03	-	1.52± 0.66	0.82± 0.42	1.98± 0.89
		2	0.85± 0.33	-	1.33± 0.97	1.65± 0.33	2.02± 1.51
		4	0.46± 0.85	-	0.80± 1.14	0.55± 1.33	1.74± 0.68
		6	0.55± 0.85	-	0.96± 0.48	0.78± 0.62	1.38± 0.82
		8	0.48± 1.18	-	1.33± 0.85	0.48± 1.02	1.60± 0.72
B	DXM	1	0.76± 0.95	-	1.14± 0.88	0.85± 0.33	1.72± 1.15
		2	0.51± 0.86	-	1.21± 0.86	-	0.94± 0.82
		4	1.05± 0.51	-	0.65± 0.55	0.48± 1.02	1.25± 0.33
		6	0.92± 0.33	-	0.57± 0.94	0.64± 0.89	1.04± 0.51
		8	0.55± 0.85	-	0.42± 1.10	-	0.72± 0.37
C	PBS	1	-	-	-	-	-
		2	-	-	-	-	-
		4	-	-	-	-	-
		6	-	-	-	-	-
		8	-	-	-	-	-

*virus titer below the detection level (<10 p.f.u. per organ)
†CPS-cyclophosphamide, DXM- dexamethasone, PBS-phosphate buffered saline

Table 6. Virus reactivation (at day 8 post-treatment) in mouse tissues for a period of eight months after a single exposure to TK-gene-deleted PrVs

Group	Drug treatment	Month p.i.	Virus isolation				
			Nasal turbinate	Lung	Cerebrum	Cerebellum	Trigeminal ganglia
D	CPS†	1	-*	-	1.33± 0.55	-	1.22± 0.47
		2	-	-	0.92± 0.37	-	1.46± 1.35
		4	-	-	1.00± 1.15	-	0.98± 1.20
		6	-	-	0.75± 0.85	-	1.05± 0.91
		8	-	-	0.84± 1.26	-	0.76± 1.18
E	DXM	1	-	-	0.69± 1.15	-	1.02± 1.19
		2	-	-	1.09± 1.21	-	0.96± 0.29
		4	-	-	0.55± 0.38	-	0.84± 0.38
		6	-	-	0.39± 0.67	-	0.42± 0.85
		8	-	-	0.42± 0.85	-	0.64± 0.28
F	PBS	1	-	-	-	-	-
		2	-	-	-	-	-
		4	-	-	-	-	-
		6	-	-	-	-	-
		8	-	-	-	-	-

*virus titer below the detection level (<10 p.f.u. per organ)
†CPS-cyclophosphamide, DXM-dexamethasone, PBS-phosphate buffered saline

Table 7. Detection of DNA of reactivated PrVs (at day 8 post-treatment) in mouse tissues by PCR for 8 month period after a single exposure to TK-gene-deleted PrV

Group	Drug treatment	Virus	Month p.i.	PCR detection				
				Nasal turbinate	Lung	Cerebrum	Cerebellum	Trigeminal ganglia
A	CPS†	Wild- type	1	4/4	2/4	4/4	3/4	4/4
			2	4/4	-	4/4	4/4	4/4
			4	4/4	1/4	4/4	3/4	4/4
			6	4/4	-	4/4	3/4	4/4
			8	4/4	-	4/4	2/4	4/4
D	CPS	TK-gene deleted	1	-*	-	4/4	-	4/4
			2	-	-	4/4	-	4/4
			4	-	-	4/4	-	4/4
			6	-	-	4/4	-	4/4
			8	-	-	4/4	-	4/4
B	DXM	Wild- type	1	4/4	1/4	4/4	3/4	4/4
			2	4/4	-	4/4	2/4	4/4
			4	4/4	1/4	4/4	2/4	4/4
			6	4/4	-	4/4	2/4	4/4
			8	4/4	-	4/4	1/4	4/4
E	DXM	TK-gene deleted	1	-	-	4/4	-	4/4
			2	-	-	4/4	-	4/4
			4	-	-	4/4	-	4/4
			6	-	-	4/4	-	4/4
			8	-	-	4/4	-	4/4

*undetected
†CPS-cyclophosphamide, DXM-dexamethasone, PBS-phosphate buffered saline

virus replication, tissue tropism and shedding.

Table 5 shows data on virus reactivation upon treatment with cyclophosphamide and dexamethasone. Throughout a period of eight months p.i., latent wild-type PrV was successfully reactivated in nervous tissues, as examined at day 8 following the respective treatments. There was some degree of variation in titers of the virus reactivated and tissue involved where treatments with cyclophosphamide resulted in more consistent results than that of dexamethasone. No virus was detected in lung tissue regardless of the type of immunosuppresants employed, but variable results were obtained for virus detection in the cerebellum of animals treated with dexamethasone. As shown in Table 6, viruses detected were only in trigeminal ganglia and cerebrum, consistent with our studies, in animals inoculated with TK-gene-deleted PrV. By using the same samples aforementioned in the PCR technique, results obtained were of similar trend in concordance with results of virus isolation (Table 7). This indicated PrV can establish latency for a long period, as long as at least eight months following primary exposure to the virus infection, and reactivated later to produce infectious viruses. The capability of the virus to remain latent for such a long period indicated the host immunesurveillence mechanism failed to detect the virus in the latent state (Cheung, 1995).

In response to virus inoculation, animals inoculated with wild-type or TK-gene-deleted PrVs produced signif-

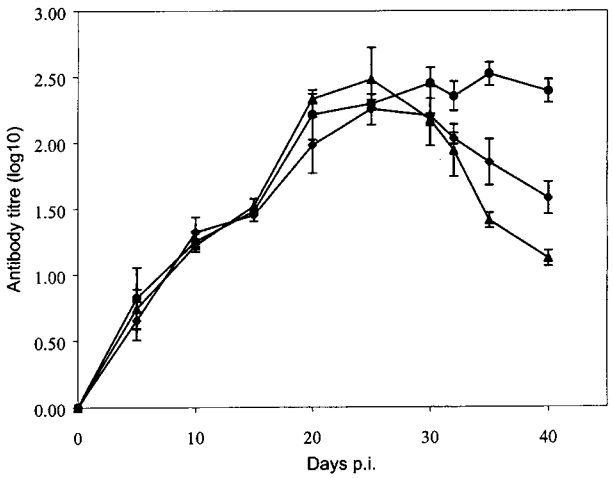

Fig. 1. Antibody titers (mean± s.d.) to wild-type PrVs prior to and upon virus activation at day 30 post virus inoculation. Each group of animals was treated with PBS (●) which served as a control, DXM(◆) and CPS(▲); n=4.

icant titers of antibody to viral antigens. Upon treatment with immunosuppressive drugs, antibody titers were reduced dramatically but the most significant reduction in titers was observed in animals treated with cyclophosphamide (Figs 1 and 2). Such differences in antibody titers could not be associated with the difference in antibody capabilities preventing virus replication since the present evidence failed to support this phenomenon. Despite remaining

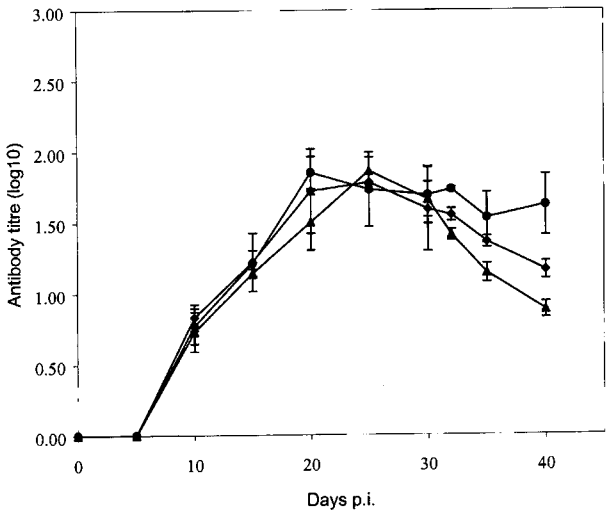

Fig. 2. Antibody titers (mean± s.d.) to TK-gene-deleted PrVs prior to and upon virus activation at day 30 post virus inoculation. Each group of animals was treated with PBS (●) which served as a control, DXM (◆), CPS (▲); n=4.

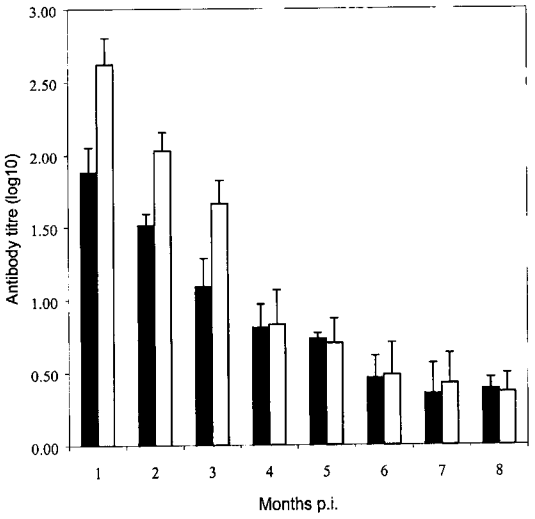

Fig. 3. Antibody titers (mean ± s.d.) to PrV antigens for a period of eight months after a single exposure to wild-type (■) and TK-gene-deleted (□) virus; n=4.

at high levels on days 2 and 5 post treatment, antibodies available did not prevent reactivation of latent virus. Furthermore, there was no evidence to indicate reduction in antibody titers throughout the eight month period following exposure to wild-type or TK-gene-deleted PrV was accompanied by a significant alteration in virus titers upon reactivation (Fig. 3). This strongly indicates other components of the immune system, which could be of T cell origin, are involved in the prevention of virus reactivation and spread. It also suggests that immunosuppressive agents may suppress T cell functions, which are responsible for the detection and destruction of virally infected cells.

Previously, PrV was claimed to establish latency in many tissues including the tonsils, lymph nodes, trigeminal ganglia (Schoenbaum *et al.*, 1990), lungs (Segales *et al.*, 1997) and nasal and buccal cavities (Cowen *et al.*, 1990). However, our studies showed that the site of latency is limited to particular tissues or cells of neural origin. Effective suppression of immune components was the main contributing factor that allows the virus to reactivate and replicate to high titers. Therefore, cyclophosphamide regimes effectively, in relation to the virus replication, reactivate the latent virus as compared to dexamethasone as previously observed in a different study (Cowen *et al.*, 1990). Although the TK-gene-deleted virus can establish latency and reactivate, the use of such virus as a vaccine may mediate a significant reduction in the virus load of wild-type PrV during challenge infection (Vilnis *et al.*, 1998). The TK-gene-deleted PrV described is suitable for the preparation of live pseudorabies vaccine, on the basis of its non-pathogencity, non-virus shedding upon restricted virus reactivation and immunogenicity. The incapability of the vaccine virus to be shed from the host would be useful for the purpose of discriminating vaccinated from non-vaccinated animals especially for disease eradication program.

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