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Encephalomalacic Lesions in Pigs Dually Infected with Porcine Reproductive and Respiratory Syndrome Virus and Pseudorabies Virus

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Summary

Four pigs (group 1) were infected with an aerosol containing porcine reproductive and respiratory syndrome virus (PRRSV) followed 7 days later by pseudorabies virus (PRV). Three further pigs (group 2) received PRRSV alone, two (group 3) received PRV alone, and two (group 4) remained as uninfected controls. Despite the admittedly small numbers of animals, the experiment appeared to throw light on aspects of synergy. Thus, the group 1 pigs showed severe neurological signs characterized by ataxia and muscular tremors. Total cell numbers in the bronchoalveolar lavage fluid were increased in all PRRSV-infected pigs, and PRRSV antigen was detected in the alveolar macrophages. Total cell numbers in the cerebrospinal fluid of group 1 pigs were considerably greater than those demonstrated in group 3, but no PRV antigen was found. Pigs of groups 1 and 2 showed pulmonary lesions, characterized by interstitial pneumonia and PRRSV antigen immunolabelling. Non-suppurative encephalitis was found in five of the six pigs of groups 1 and 3. In particular, one group 1 animal had severe necrotizing encephalitis with intranuclear inclusion bodies and associated immunolabelling of PRV antigen. The other three group 1 pigs had prominent malacic lesions, with macrophages. These neuropathological findings strongly suggested that PRRSV infection in pigs enhances the severity of brain lesions caused PRV.

Keywords: encephalomalacia; pigs; porcine reproductive and respiratory syndrome; PRRS; pseudorabies; synergy; viral infection

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an arterivirus disease, currently endemic in many swine-producing countries (Conzelmann *et al.*, 1993). PRRS virus (PRRSV) primarily infects and destroys alveolar macrophages, which play an important role in pulmonary defence (Rossow, 1998). PRRSV is therefore suspected to have an immunosuppressive effect on pigs.

Co-infection with PRRSV has been reported to increase the severity of disease produced by agents such as porcine respiratory coronavirus, influenza virus, mycobacteria, *Salmonella choleraesuis* and *Streptococcus suis* (Groschup *et al.*, 1993; Kawashima *et al.*, 1996; Reeth *et al.*, 1996; Narita *et al.*, 1997; Thacker *et al.*, 1999; De Bruin *et al.*, 2000; Thanawongnuwech *et al.*, 2000; Wills *et al.*, 2000). Such synergism has been supported by clinical, virological and immunological observations. The immunosuppressive effect of PRRSV on the host response to pseudorabies virus (PRV) has also been investigated in the respiratory system (Shibata *et al.*, 2003), but there have been few histopathological studies supporting synergism in dual infections with PRRSV and PRV, or in dual infection with PRRSV and other respiratory viral agents (De Bruin *et al.*, 2000).

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The purpose of the present study in admittedly small numbers of specific pathogen-free (SPF) pigs was to throw light on the effects (clinical, cytological and pathological) of dual infection with PRRSV and PRV as compared with single infections.

Materials and Methods

Animals

Eleven SPF pigs aged 4 weeks were used, having been shown to be free from serum antibodies against PRV, PRRSV and porcine circovirus type 2. The pigs were divided into four groups (see below), which were housed separately in different blocks to prevent cross-infection. The housing conditions included a filtered air system and regulated temperature.

Virus

The EDRD-1 strain of PRRSV, kindly provided by Dr Y. Murakami of the National Institute of Animal Health, was used after eight passages in swine alveolar macrophages. The YS-81 strain of PRV, also provided by Dr Y. Murakami, was used after three passages in pig kidney cell culture. The infected culture fluids were used as inocula.

Experimental Procedure

The pigs were randomly assigned to four groups, as follows: group 1 (pigs 1–4) were inoculated with PRRSV followed by PRV; group 2 (pigs 5–7) received PRRSV alone; group 3 (pigs 8 and 9) received PRV alone; group 4 (pigs 10 and 11) were non-infected controls. The inocula were administered as an aerosol produced by a nebulizer, after the animals had been anaesthetized with ketamine hydrochloride (3 mg/kg body weight) and xylazine (2 mg, intramuscularly). On "day 0" of the experiment, pigs of groups 1 and 2 received 105.0 $TCID_{50}$ of PRRSV in a dose volume of 3 ml. On day 7 of the experiment, the pigs of groups 1 and 3 received 105.0 $TCID_{50}$ of PRV in a dose volume of 2 ml. The negative control animals of group 4 received 3 ml of non-infected culture medium on day 0, and 2 ml on day 7. All animals were killed with an intravenous overdose of pentobarbital sodium on the following days of the experiment: day 7, pig 5; day 14, pigs 1, 6, 8 and 10; and day 21, pigs 2, 3, 4, 7, 9 and 11.

Analysis of Cerebrospinal Fluid (CSF) and Bronchoalveolar Lavage Fluid (BALF)

A CSF sample (2 ml) was collected by syringe *post mortem* and placed in a sterile bottle. After removal of the lungs, a cannula (4 mm in diameter) was inserted into the right main bronchus, and 20 ml of sterile phosphate-buffered saline (PBS) were introduced into the bronchus and then recovered by suction. Counts of nucleated cells per microlitre of CSF and BALF were made, and 0.2 ml of CSF and 0.2 ml of a 1 in 10 dilution of BALF were centrifuged and stained with a Diff-Quik Kit (International Reagent Corporation, Koube, Japan). Evaluation included a 200-cell differential count and a morphological description of the cells. The centrifuged CSF and BALF cells were fixed in cold acetone and stored at −20 °C for immunohistochemical examination.

Histopathology and Immunohistochemistry

Specimens from each pig, including parts of the brain (cerebral cortex, frontal lobe, motor area, occipital lobe, corpus striatum, thalamus, colliculus caudalis, cerebellar peduncles, pons, cerebellum and medulla oblongata), spinal cord, trigeminal ganglia, tonsil and lung, were fixed in 10% neutral buffered formalin. The tissues were embedded in paraffin wax, sectioned (4 μm), stained with haematoxylin and eosin (HE), and examined by light microscopy.

PRRSV and PRV antigen in the formalin-fixed tissues and cold acetone-fixed CSF and BALF cells were demonstrated by the streptavidin–biotin (SAB) immunoperoxidase (IP) method, with a Histofine SAB Kit (Nichirei Corp., Tokyo, Japan). Anti-PRRSV (Chiba 92-1 strain) rabbit serum (provided by Dr K. Kawashima, National Institute of Animal Health, Japan) (Kawashima *et al.*, 1996) and anti-PRV rabbit serum (provided by Dr T. Imada, National Institute of Animal Health, Japan) (Narita *et al.*, 1985, 1991) were used as the primary antibodies at dilutions of 1 in 8000 and 1 in 2048, respectively. Sections were counterstained with methyl green. Tissue sections from non-infected control pigs (nos 10 and 11) and serum from a non-immunized rabbit were used for control purposes.

Results

Clinical Signs

All four pigs dually infected with PRRSV and PRV (group 1) showed severe neurological signs, characterized by ataxia and muscular tremors, after being infected with PRV, and had a severe febrile response (up to 40 °C) between days 1 and 10. The three group 2 pigs, infected with PRRSV

Table 1
Counts* and viral antigen immunolabelling of cells in the CSF and BALF collected *post mortem* from pigs of groups 1–4

	CSF and [BALF] results in individual pigs of										
	group 1				*group 2*			*group 3*		*group 4*	
Observation	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
Total cells (× 103.0)	43 [58]	32 [113]	nd [52]	66 [146]	5 [33]	8 [56]	9 [178]	29 [42]	nd [30]	6 [24]	5 [29]
Neutrophils (%)	2 [4]	8 [14]	nd [3]	0 [30]	0 [8]	0 [19]	0 [9]	8 [3]	Nd [12]	6 [3]	5 [2]
Macrophages (%)	81 [9]	74 [42]	nd [55]	80 [48]	0 [80]	0 [58]	0 [45]	76 [91]	nd [71]	0 [83]	0 [88]
Lymphocytes (%)	12 [6]	10 [40]	nd [40]	14 [18]	0 [8]	0 [20]	0 [44]	8 [4]	nd [14]	0 [9]	0 [6]
PRRSV antigen	– [+]	– [+]	nd [−]	– [+]	– [−]	– [−]	– [+]	– [−]	nd [−]	– [−]	– [−]
PRV antigen	– [−]	– [−]	nd [−]	– [−]	– [−]	– [−]	– [−]	– [−]	nd [−]	– [−]	– [−]

nd, Not done. +, A few immunolabelled cells; −, none. See Materials and Methods for details of inoculation of groups 1–4. The experiment began on "day 0" and the CSF and BALF were collected *post mortem* at the following times: pig 5 (day 7); pigs 1,6,8 and 10 (day 14); pigs 2,3,4,7,9 and 11 (day 21).
* × 103.0 for CSF, but × 104.0 for BALF.

alone, showed slight respiratory symptoms and had a transient (2-day) febrile response. The two group 3 pigs, infected with PRV alone, showed inappetence and pyrexia for a period of 5 days. The two non-infected control pigs (group 4) showed no clinical abnormalities during the experimental period.

Cytology

CSF cells. The results are shown in Table 1. The total cell number in the CSF from pigs infected with PRV (groups 1 and 3) ranged from 29 to 66 (× 103.0)/ml. The proportions of the various cell types were: neutrophils 2–8%, macrophages 74–81% and lymphocytes 8–14%. The total cell numbers of pigs infected with PRRSV alone (group 2) and non-infected controls (group 4) ranged from 5 to 9 (× 103.0)/ml and 5 to 6 (× 103.0)/ml, respectively, and no neutrophils or macrophages were found. Despite the small numbers of observations, there was an indication that the total number of CSF cells may have been considerably higher in group 1 than in group 3. No PRV or PRRSV antigen was detected by immunolabelling of the CSF cells of any animals.

BALF cells. The total cell number in the BALF from pigs infected with PRRSV (groups 1 and 2) ranged from 33 to 178 (× 104.0)/ml. The proportions of the various cell types were: neutrophils 3–30%, macrophages 9–80%, and lymphocytes 6–44%. The total cell number in pigs infected with PRV alone (group 3) was 30 to 42 (× 104.0)/ml, and in non-infected control pigs (group 4) was 24 to 29 (× 104.0)/ml. The proportion of neutrophils in pigs infected with PRV alone (group 3) was 3–12%, that of macrophages was 71–91%, and that of lymphocytes was 4–14%. In non-infected control pigs (group 4), the corresponding proportions were as follows: neutrophils 2–3%, macrophages 83–88%, and lymphocytes 6–9%. There was no obvious difference between groups 1 and 2. PRRSV (but not PRV) antigen was detected in the BALF macrophages of four the seven pigs infected with PRRSV (groups 1 and 2) (Fig. 1).

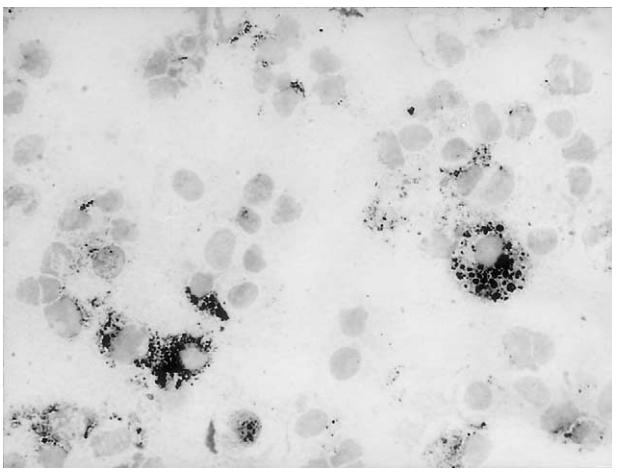

Fig. 1. PRRSV antigen in the BALF cells of a dually infected pig (no. 1). SAB-IP. × 400.

Table 2
Distribution of histopathological lesions in the central nervous system (CNS) of pigs infected dually and singly with PRRSV and PRV

	Encephalitis/Encephalomalacia* in individual pigs of*										
	group 1				*group 2*			*group 3*		*group 4*	
CNS site	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
Cerebrum											
Frontalis	+++/−	+/+++	+/+++	+/+++	−/−	−/−	−/−	+/−	−/−	−/−	−/−
Temporalis	++/−	+/++	+/++	+/++	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Parietalis	+/−	+/+	+/++	+/++	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Occipitalis	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Hippocampus	+/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Thalamus	+/−	+/−	+/−	+/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Cerebellum	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Pons	+/−	+/−	+/−	+/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Medulla oblongata	+/−	+/−	+/−	+/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Trigeminal ganglia	+/−	+/−	+/−	+/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Spinal cord	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−

See Materials and Methods for details of inoculation of groups 1–4, and for times at which the animals were killed for examination. Interstitial pneumonia: moderate (pigs 1, 2, 3, 6, 7), slight (pig 4), negative (pigs 5, 8, 9, 10,11). Tonsillitis: severe (pig 1), moderate (pigs 2, 3, 4), slight (pig 9); negative (pigs 5, 6, 7, 8, 10, 11). Thymic atrophy: severe (pigs 1–4), slight (pigs 8, 9), negative (pigs 5, 6, 7, 10, 11).
* − , Negative; + , slight; ++, moderate; +++, severe.

Pathology

Macroscopically, all dually infected pigs (group 1) showed severe atrophy of the thymus, congestion of the brain, and pneumonia with diffuse tan coloration at the periphery of the lobes. None of the three pigs infected with PRRSV alone (group 2) or the two pigs infected with PRV alone (group 3) showed obvious macroscopical lesions, except for slight atrophy of the thymus in the group 3 animals. The two non-infected control pigs (group 4) showed no abnormalities.

The distribution of microscopical lesions is summarized in Table 2. All dually infected pigs (nos 1–4) had slight trigeminal ganglioneuritis and slight (pigs 2–4) to severe (pig 1) non-suppurative encephalitis, consisting of neuronal degeneration and necrosis, neuronophagia, diffuse or focal glial reactions, and perivascular cuffing (Fig. 2). The lesions were distributed in the white matter of the lobus frontalis, lobus temporalis and lobus parietalis, but not in the cerebellum or spinal cord. The more severe lesions occurred in the frontalis areas of the cerebral grey matter. Some degenerating neuronal and glial cells adjacent to the necrotic areas showed basophilic intranuclear inclusion bodies. Three dually infected pigs (nos 2–4) killed on day 21 of the experiment, had prominent encephalomalacic lesions, localized in

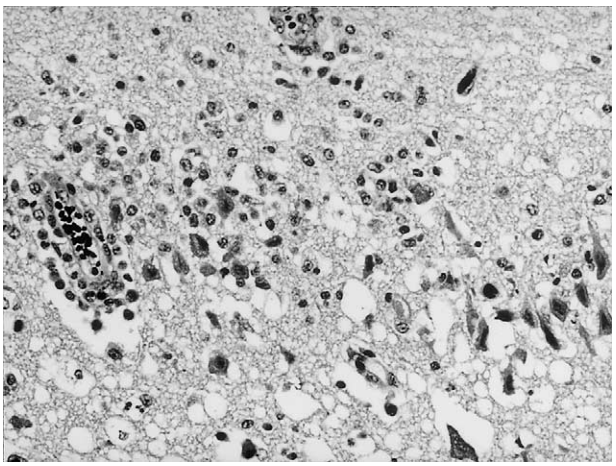

Fig. 2. Non-suppurative encephalitis consisting of neuronal degeneration, a diffuse glial reaction and perivascular cuffings in the lobus frontalis of a dually infected pig (no. 1). HE. × 200.

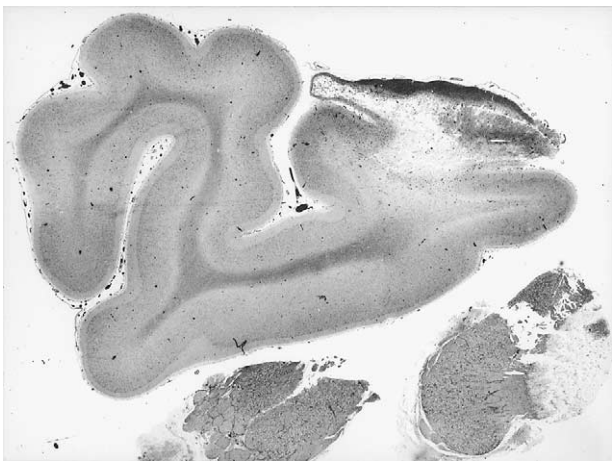

Fig. 3. Encephalomalacic lesion in the olfactory tract of a dually infected pig (no. 3). HE. × 2.1.

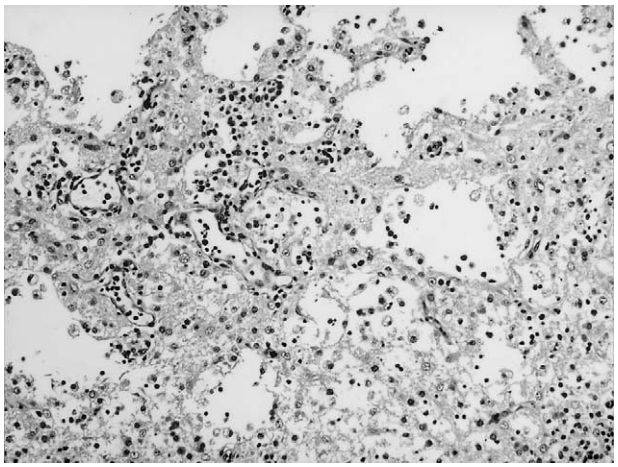

Fig. 4. Cortical cavitation with macrophages in the lobus temporalis of a dually infected pig (no. 2). HE. × 100.

areas near the olfactory bulb and rhinencephalon, including the olfactory tract (Fig. 3), olfactory stria, and pyriform cortex. They consisted of cortical cavitation, with macrophages (Fig. 4). Pig 8, infected with PRV alone, showed slight perivascular cuffing and glial reaction but no neuronal degeneration or intranuclear inclusion bodies.

The four group 1 pigs and two of the three group 2 pigs had slight to moderate interstitial pneumonia (Fig. 5), but no necrotizing bronchiolitis. All group 1 pigs had moderate tonsillitis with typical intranuclear inclusion bodies in the degenerating crypt epithelial cells; they also had severe atrophy of the thymus with apoptosis of thymic T lymphocytes (Fig. 6). Two group 3 pigs also had slight thymic atrophy. Such lesions were not observed in the pigs of groups 2 and 4.

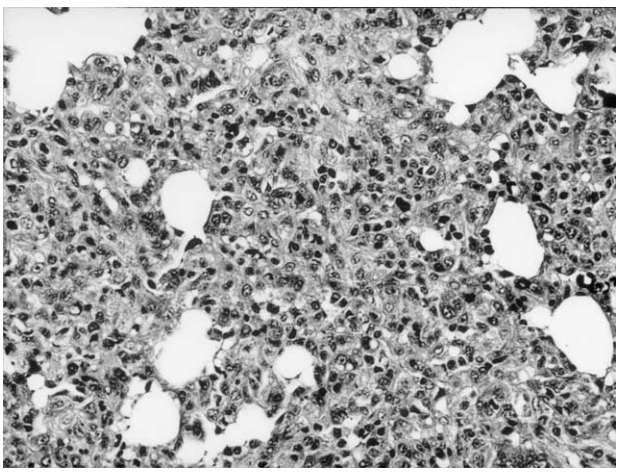

Fig. 5. Moderate interstitial pneumonia in a dually infected pig (no. 3). HE. × 200.

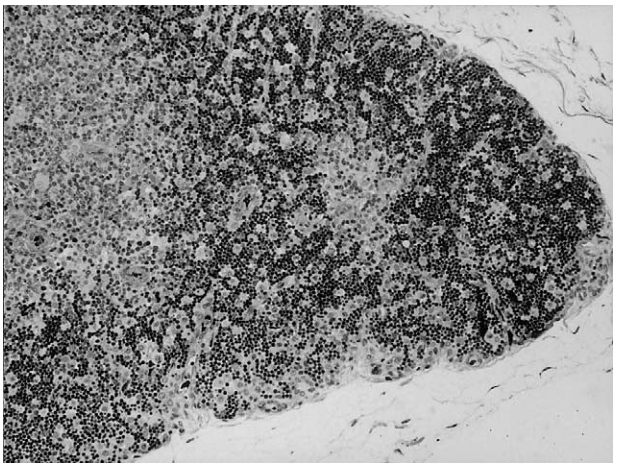

Fig. 6. Atrophy of thymus with apoptosis of thymic T lymphocytes in a dually infected pig (no. 2). HE. × 100.

Immunohistochemistry

The distribution of viral antigens in tissues is summarized in Table 3. In the cerebrum, PRV antigen was found in three (nos 1,2 and 4) of the dually infected pigs. Strong immunolabelling was closely associated with the intranuclear inclusion bodies in the neuronal cells of pig 1, killed on day 14 of the experiment (Fig. 7). Immunolabelling was observed in a few glial cells of pigs 2 and 4 but not in the macrophages in the encephalomalacic lesions. In the lungs of pigs 2–7, PRRSV antigen (but not PRV antigen) was detected in macrophages in the alveolar lumina at sites showing interstitial pneumonia (Fig. 8). PRV antigen was detected in the degenerating epithelial cells of the tonsillar crypts of the four dually infected pigs, and PRRSV antigen was detected in the macrophages of two of them (nos 3 and 4) and one pig (no. 6) that received PRRSV alone. Neither antigen was found in the thymus of any pig or in any tissue from the two non-infected control pigs.

Discussion

The synergistic effect of dual infection with PRRSV and other infectious agents has been investigated previously (Groschup *et al.*, 1993; Kawashima *et al.*, 1996; Reeth *et al.*, 1996; Narita *et al.*, 1997, 2000; Thacker *et al.*, 1999; De Bruin *et al.*, 2000; Thanawongnuwech *et al.*, 2000; Wills *et al.*, 2000). In this study, pigs infected with PRRSV or PRV alone did not show severe clinical signs, but there was a febrile response in pigs infected with PRV, and there were slight respiratory symptoms in pigs infected with PRRSV. The four dually infected pigs

Table 3
Distribution of viral antigen immunolabelling in pigs infected dually or singly with PRRSV and PRV

	Immunolabelling for PRV [or PRRSV] antigens in individual pigs of*										
	group 1				*group 2*			*group 3*		*group 4*	
Site	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
Cerebrum	+++	+	−	+	−	−	−	−	−	−	−
	[−]	[−]	[−]	[−]	[−]	[−]	[−]	[−]	[−]	[−]	[−]
Trigeminal ganglia	+	−	−	−	−	−	−	−	−	−	−
	[−]	[−]	[−]	[−]	[−]	[−]	[−]	[−]	[−]	[−]	[−]
Lung	−	−	−	−	−	−	−	−	−	−	−
	[−]	[+]	[++]	[+]	[+]	[+]	[+]	[−]	[−]	[−]	[−]
Tonsil	++	++	+	+	−	−	−	−	−	−	−
	[−]	[−]	[+]	[+]	[−]	[+]	[−]	[−]	[−]	[−]	[−]
Thymus	−	−	−	−	−	−	−	−	−	−	−
	[−]	[−]	[−]	[−]	[−]	[−]	[−]	[−]	[−]	[−]	[−]

See Materials and Methods for details of inoculation of groups 1–4, and for times at which animals were killed for examination.
*Number of immunolabelled cells: −, none; +, few; ++, moderate; +++, many.

(group 1), however, showed severe neurological signs characterized by ataxia and muscular tremors. These findings, which resembled those reported by Shibata *et al.* (2003), suggested that PRRSV infection in pigs affects the replication of PRV.

Each of the infectious agents produces characteristic lesions at the various sites of viral replication (Narita *et al.*, 1985, 1991, 1993; Rossow, 1998). In the present experiment, seven pigs infected with PRRSV (groups 1 and 2) had slight interstitial pneumonia but no necrotizing bronchiolitis. These pneumonic lesions were closely associated with the presence of PRRSV antigen, and were probably due to PRRSV infection since they resembled PRRSV-associated lesions described in previous reports (Rossow, 1998; Shibata *et al.*, 2003). Five of six pigs infected with PRV (groups 1 and 3) had non-suppurative encephalitis and trigeminal ganglioneuritis. One dually infected pig (no. 1) had severe necrotizing encephalitis with intranuclear inclusion bodies, while the other three (nos 2–4) had prominent encephalomalacia, with macrophages. All dually infected pigs had tonsillitis with inclusion bodies and severe atrophy of the thymus. These results strongly suggest that PRRSV infection in pigs can increase the severity of brain and tonsillar lesions due to PRV replication, and also the severity of lung lesions (Shibata *et al.*, 2003). Moreover, thymic atrophy may be related to dual infection.

The total number of CSF cells increased 4- to 8-fold in PRV-infected pigs as compared with the numbers in pigs infected with PRRSV alone and in the non-infected pigs. Moreover, the total number

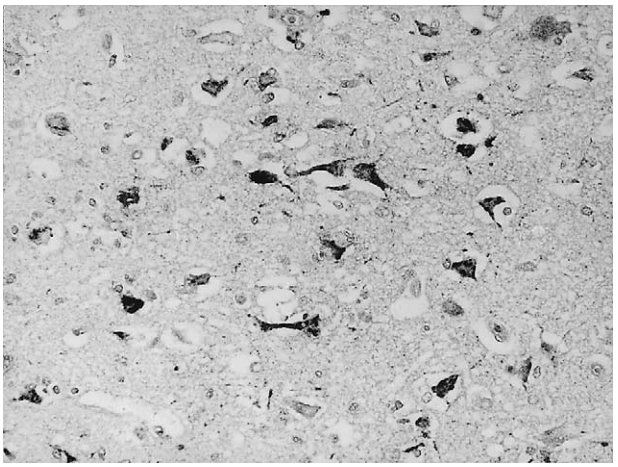

Fig. 7. PRV antigens in the degenerated neuronal cells of a dually infected pig (no. 1). SAB-IP. × 200.

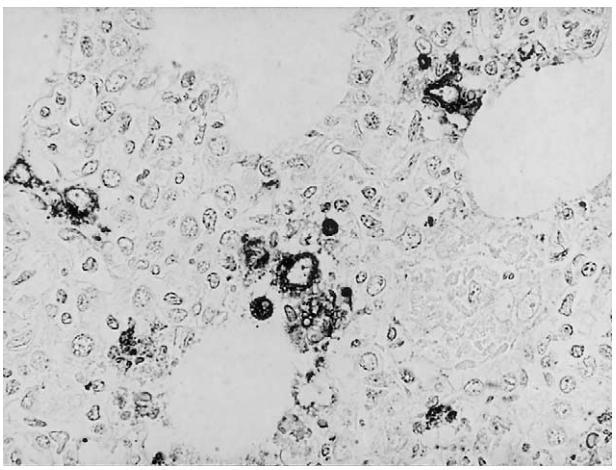

Fig. 8. PRRSV antigens in the pulmonary macrophages of a pig (no. 5) infected with PRRSV alone. SAB-IP. × 400.

was higher in dually infected pigs that in those infected with PRV alone. In PRV-infected pigs, neither PRV nor PRRSV antigen was detected in CSF cells. The total cell number in the BALF increased by up to 4-fold in all PRRSV-infected pigs, and PRRSV (but not PRV) antigen was detected in the alveolar macrophages. These results corresponded well with the presence of interstitial pneumonia. Thus, the results of CSF cell analysis accorded with the severity of the brain lesions in the dually infected pigs, and the results of BALF cell analysis with the interstitial pneumonic lesions in PRRSV-infected pigs.

Herpes viruses spread via sensory axons and infect sensory neurons in the ganglia of the peripheral nervous system (Cook and Stevens, 1973; Narita *et al.*, 1991, 2001; Chowdhury *et al.*, 1997; Yanai *et al.*, 2003). In PRV infection in pigs, infection spreads from the nasal cavity via the olfactory pathway to produce non-suppurative encephalitis (Narita *et al.*, 1985, 1991, 1992, 1993, 1997). In the present experiments, one dually infected pig had severe encephalitis and three had encephalomalacia, characterized by neuronal degeneration with intranuclear inclusion bodies, a diffuse or focal glial reaction, and perivascular cuffing, as well as by cavitation accompanied by large numbers of macrophages. The lesions were mainly confined to the olfactory bulb and rhinencephalon, including the olfactory tract, olfactory stria, and pyriform cortex. The encephalomalacic lesions may therefore have developed from necrotic foci similar to those observed in PRV-infected newborn piglets (Nunoya *et al.*, 1985) and in animals with fluctuating temperatures (Narita *et al.*, 1991). Thus, the neuropathological findings strongly suggested that PRRSV infection in pigs enhanced the severity of brain lesions caused by PRV. This apparent synergy was supported by histopathological findings.

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