In vivo Replication of Porcine Reproductive and Respiratory Syndrome Virus in Swine Alveolar Macrophages and Change in the Cell Population in Bronchoalveolar Lavage Fluid after Infection

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ABSTRACT. Replication of porcine reproductive and respiratory syndrome (PRRS) virus in swine alveolar macrophages (AM) and cell population in broncho-alveolar lavage fluid (BALF) obtained from PRRS virus-infected pigs were investigated. BALF samples were periodically collected from 6 pigs infected with PRRS virus and 3 non-inoculated control pigs by means of fiber-optic bronchoscope between post-inoculation day (PID) 0 and 56. The mean ratio of macrophages in BALF collected from infected group was 92.7 ± 3.2% before inoculation and gradually decreased from PID 14. On the other hand, the ratio of lymphocytes was 4.8 ± 3.2% before inoculation and increased from PID 21 and indicated 41.8 ± 9.1% on PID 28. After that, they decreased gradually and that of macrophages correspondingly increased. The ratio of neutrophils maintained between 0.7% and 5.1%. The ratios of macrophages, lymphocytes and neutrophils collected from control group were almost stable through the examination. Intracellular PRRS virus antigens in AM were detected from PID 2 by indirect immunofluorescence assay (IIFA). PRRS virus was first isolated from BALF samples collected from inoculated group between PID 2 and 49. From serum, virus was isolated between PID 2 and 21. Antibodies in sera measured by IIFA to PRRS virus were first detected on PID 14 and the antibody titer rose to 1:640 or 1: 1,280. The results suggested that PRRS virus replicates in swine AM for a relatively long period. — KEY WORDS: alveolar macrophage, broncho-alveolar lavage, PRRS virus, swine.

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Porcine reproductive and respiratory syndrome (PRRS) was recognized in North America in 1987 [6] and thereafter in Europe in 1990s [9, 19]. PRRS is mainly characterized by reproductive disturbance in sows and respiratory distress in young pigs. The causative agent was first isolated in swine alveolar macrophages (AM) and named Lelystad virus [18]. Shortly thereafter, similar viruses were isolated in many countries. In Japan, PRRS viruses were first isolated from fattening pigs and stillborn pigs in 1993 [8, 12, 16]. The antigenicity of the Japanese viruses was more closely related to that of American type virus than to that of European type virus [12, 15]. AM are believed to be a target cell for PRRS virus replication *in vivo* [14]. Mengeling *et al.* [11] reported that PRRS virus was isolated from AM for several weeks after infection.

This paper describes the *in vivo* replication of PRRS virus in AM of infected pigs. Furthermore, cell population in broncho-alveolar lavage fluid (BALF) after infection was also examined, because alveolar lavage samples are suitable to study inflammatory and immune response in the alveolar structures [5].

MATERIALS AND METHODS

Experimental infection: Six of 9 4-week-old specific pathogen-free (SPF) pigs were inoculated intranasally with $10^{4.8}$ $TCID_{50}$ of E4 strain [15] of PRRS virus and the remaining three pigs served as non-inoculated control. The two groups were housed separately in barrier-maintained rooms.

Broncho-alveolar lavage: AM were periodically collected from live pigs by broncho-alveolar lavage between on post-inoculation day (PID) 0 and 56. Segmental broncho-alveolar lavage was done as follows. The pigs were anesthetized by intramuscular injection of ketamine (Sankyo Inc.) after being preanesthetized by azaperon (Sankyo Inc.) and atropine sulfate (Tanabe Inc.). Fiber-optic bronchoscope (BF type P20, Olympus, Japan) was inserted into the bronchus of frontal pulmonary lobe and 40 m*l* of Hanks' solution were infused and aspirated immediately.

Cytological evaluation of BALF: BALF was washed with Hanks' solution two times and adjusted to 10^6 cells/m*l*. Cytospin preparations (Cytospin 2, Shandon Southern Products Ltd, England) of each recovered sedimented cells were fixed and stained with Wright Giemsa stain. Evaluation included a 200-cell defferential count and morphologic description of cells.

Virus isolation and detection: BALF was centrifuged at 1,500 rpm for 10 min. The supernatant fluid was centrifuged again at 3,000 rpm 15 min and stored at −80°C until virus isolation (SF sample). The sedimented cells were washed three times with RPMI 1640 medium containing 10% fetal calf serum and antibiotics (PRMI-FCS). The cell numbers were finally adjusted to 5×10^6 cells/m*l* in PRMI-FCS and stored at −80°C (AM sample). AM samples for virus isolation were thawed, sonicated and centrifuged. The supernatants were used for virus isolation. Virus isolation of AM samples, SF samples and sera was performed by the microtiter method using swine AM cell cultures [4].

For detection of virus antigen in AM indirect immunofluorescent assay (IIFA) was carried out as follows. Acetone-fixed AM samples which were air-dried on a slide glass were stained with convalescent SPF pig serum with E4 strain as primary antibody at 37°C for 50 min. After

incubation, they were washed three times with PBS and stained with protein A conjugated with fluorescein isothiocyanate (Zymed, U.S.A.) with same conditions. They were washed three times with PBS and examined under a fluorescent microscope.

Serology: For serological examination, IIFA was carried out according to the previous report [4] with some modifications. Fixed MARC-145 cell [7] cultures grown on cover glass infected with EDRD-1 strain [12] were used for IIFA antigen. Protein A conjugated with fluorescein isothiocyanate was used instead of rabbit anti-swine IgG conjugated with the same dye (Samegai *et al.* at the 118th meeting of the Japanese Society of Veterinary Science in 1994). Test sera were diluted two-fold serially from 1:20 to 1:2,560.

RESULTS

In the pigs inoculated with PRRS virus no clinical signs of illness were observed except a febrile response for two weeks after inoculation.

The transitions in ratios of macrophage, lymphocyte and neutrophil are shown in Figs. 1 and 2. Morphologically, before inoculation with PRRS virus, the mean ratio of macrophages in broncho-alveolar lavage cells collected from pigs of infected group were 92.7 ± 3.2% (Fig. 3a), and decreased from PID 14 (Fig. 1). They were 55.9 ± 7.2%, 54.3 ± 8.6% and 60.3 ± 11.5% on PID 21, 28 and 35, respectively (Fig. 3b), and after that, gradually increased. The mean ratio of lymphocytes was 4.8 ± 3.2% before inoculation, and increased from PID 21 (Fig. 1). They were 40.4 ± 6.1%, 41.8 ± 9.1% and 34.6 ± 11.4% on PID 21, 28 and 35, respectively, and they gradually decreased thereafter. The ratio of neutrophils maintained between 0.7% and 5.1% throughout the observation period. The ratios of macrophages, lymphocytes and neutrophils collected from control group were at start 97.5 ± 0.3%, 1.4 ± 0.6% and 0.8 ± 0.5%, respectively and each ratio was almost stable through the examination period (Fig. 2).

Intracellular PRRS virus antigens in AM were first detected on PID 2 and relatively many positive cells were observed between PID 7 and 21 (data not shown) (Fig. 4). PRRS virus was isolated from AM and SF samples collected from all 6 pigs inoculated with PRRS virus (Table 1). On PID 2, virus was first isolated from AM and/or SF samples of 4 pigs, and isolated from all pigs on PID 7 to 21. Thereafter, virus was also isolated from AM and / or SF samples of 4 pigs between PID 28 and 35 and last isolated from pig Nos. 1 and 6 on PID 49. The AM or SF samples showed highest virus titers on PID 7 or 14. Virus titers of SF samples were usually higher than that of AM samples. From serum, virus was first isolated on PID 2 from all infected pigs and thereafter, usually isolated between PID 7 and 21 (Table 1).

All pigs were free of IIFA antibody for PRRS virus before inoculation. IIFA antibodies were first detected in the sera from pigs on PID 14 with antibody titers of 40 to 160 (Table 2). Titers of antibody reached their maximum on PID 35 to 42 and then gradually decreased later. Control pigs were free of antibody through the examination.

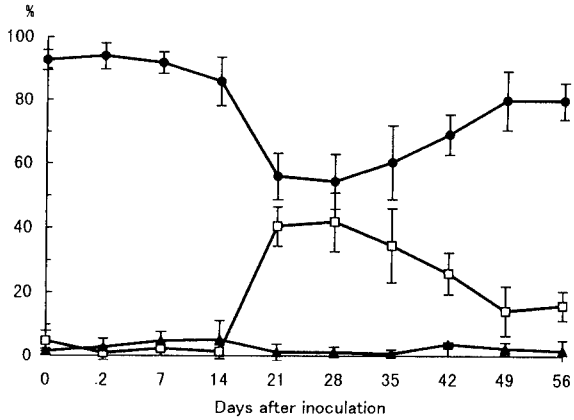

Fig. 1. Mean ratios of macrophage (—●—), lymphocyte (—□—) and neutrophil (—▲—) in broncho-alveolar lavage fluid collected from pigs inoculated with porcine reproductive and respiratory syndrome virus.

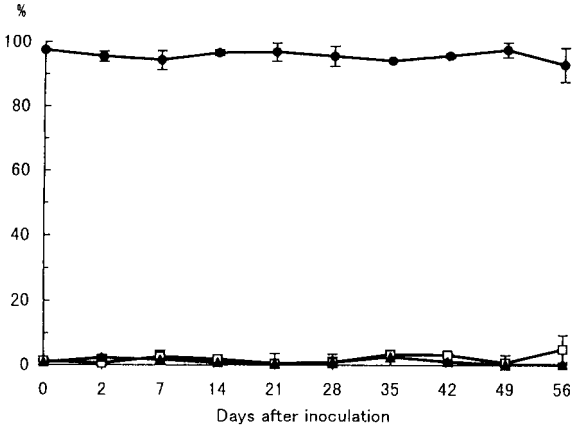

Fig. 2. Mean ratios of macrophage (—●—), lymphocyte (—□—) and neutrophil (—▲—) in broncho-alveolar lavage fluid collected from non-inoculated control pigs.

DISCUSSION

BALF samples were periodically collected from pigs infected with PRRS virus by broncho-alveolar lavage using bronchoscope. The repeated broncho-alveolar lavage had minimal effects on any of the variables examined in this study, because the populations of the BALF cells collected from control pigs were almost stable throughout the observation period. The similar result was reported in another study [11].

Differential cell counts of BALF are useful indicators of pulmonary inflammation [2, 5, 13]. The ratio of lymphocytes in lavage fluid cells increased after PRRS virus infection. This phenomenon may be the result of the

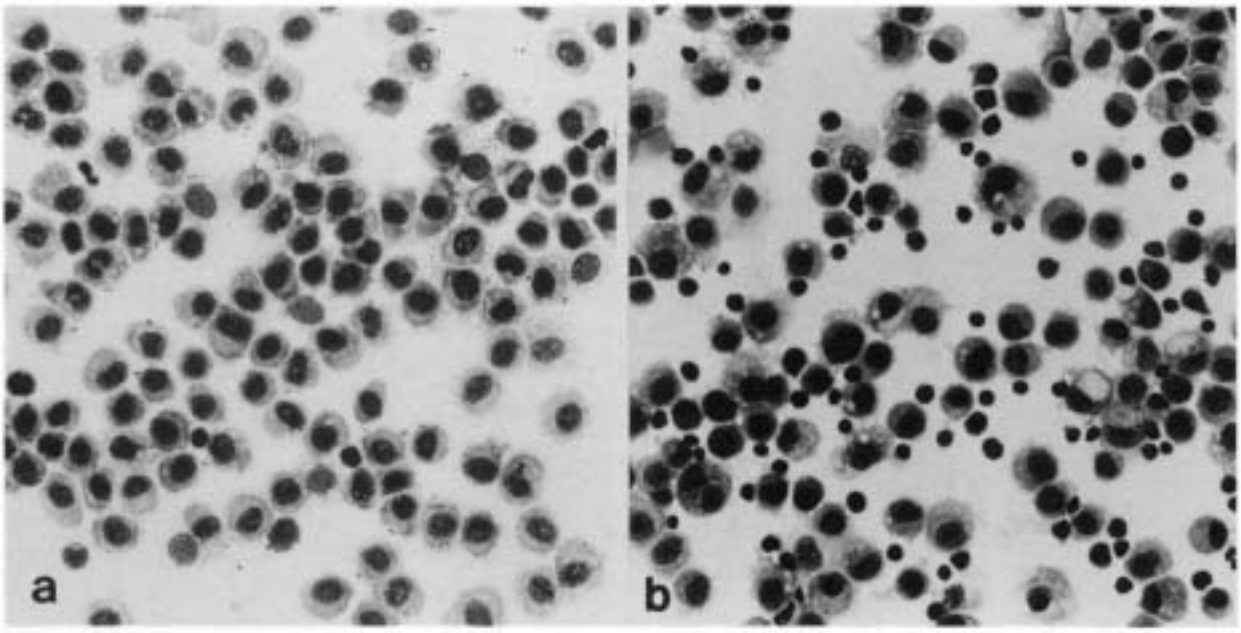

Fig. 3. Sedimented cells collected from pig inoculated with porcine reproductive and respiratory syndrome virus by broncho-alveolar lavage on post-inoculation day 0 (a) and 21 (b). Wright Giemsa stain.

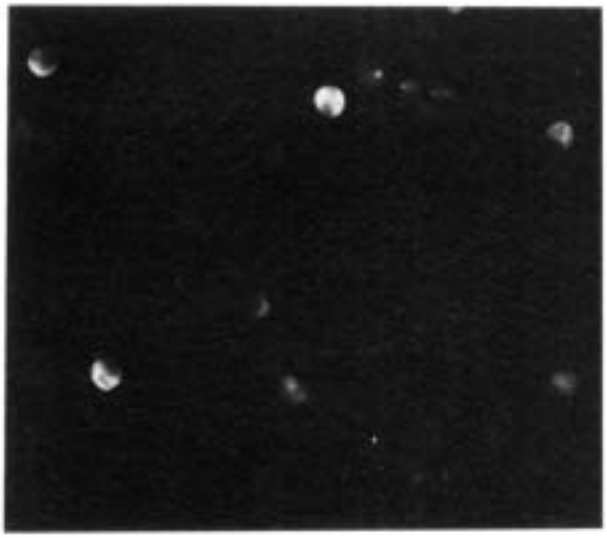

Fig. 4. Immunofluorescence staining of sedimented cells collected from pig inoculated with porcine reproductive and respiratory syndrome virus by broncho-alveolar lavage on post-inoculation day 21. Specific antigens are observed in alveolar macrophages.

immune response against PRRS virus replication in the lungs. Christianson *et al*. [1] reported that peripheral blood lymphocytes in the PRRS virus inoculated sows apparently decreased between PID 3 and 11, and the CD4+/CD8+ peripheral blood lymphocytes ratio decreased. However, there are no investigations concerning the cell population in BALF periodically collected from pigs infected with PRRS virus. On the other hand, Zhou *et al*. (In: American Association Swine Pract. Newsl. Int. PRRS Symposium Edition, 1992) reported that high levels of interleukin 1 are expressed in AM of PRRS virus-infected pigs. Consequently, it is interesting to analyze the transition of lymphocyte subset and cytokine production in lavage fluid after PRRS virus infection for the investigation of local immunity in the lung.

The result that PRRS virus was isolated from AM or SF for a longer period than from serum after inoculation would indicated that virus in alveoli was less affected than that in serum with circulating antibodies. AM play an important role in protecting the lungs against infection, and provide an important first line of defense against respiratory pathogens. Impairment of AM functions after viral infection are associated with predisposition to secondary pulmonary infection. The severity of experimentally reproduced clinical signs and lesions with PRRS virus alone were generally mild to moderate [4]. It has been demonstrated that concurrent infection with PRRS virus and porcine respiratory coronavirus infection or swine influenza virus [17], or PRRS virus and *Streptococcus suis* [3] can induce more severe disease than that with PRRS virus alone. It is possible to speculate that at least during period of virus infection in AM, the defense in the lung is suppressed and the susceptibility of an infected animal to secondary bacterial or virus infection is increased. Further investigations whether PRRS virus infection in AM affects on the cell function, such as phagocytosis are required.

PRRS viruses were isolated from AM and/or SF samples collected between PID 2 and 49 and sera collected between PID 2 and 21. As a diagnostic sample, serum and BALF sample were suitable for detecting early infection, and BALF sample was the better samples for later stages of infection,

Table 1. Virus isolation from broncho-alveolar lavage fluid samples collected from pigs inoculated with porcine reproductive and respiratory syndrome virus

Group	Pig no.	Sample[a]	Days after inoculation									
			0	2	7	14	21	28	35	42	49	56
Inoculated	1	AM	–[b]	–	2.75[c]	1.75	2.25	0.5	+[d]	–	–	–
		SF	–	+	≧3.5	≧3.25	1.75	–	+	1.25	+	–
		Serum	–	2.75	2.05	+	–	–	–	–	–	–
	2	AM	–	+	1.5	2.0	0.75	–	–	–	–	–
		SF	–	0.5	2.75	3.0	1.25	–	+	–	–	–
		Serum	–	+	+	1.8	–	–	–	–	NT[e]	–
	3	AM	–	–	2.75	1.5	+	+	–	–	–	–
		SF	–	–	≧3.5	2.5	+	–	–	–	–	–
		Serum	–	2.0	2.05	2.55	+	–	–	–	NT	–
	4	AM	–	–	3.0	1.0	–	–	–	–	–	–
		SF	–	–	3.0	3.0	+	–	–	–	–	–
		Serum	–	2.75	1.8	1.8	+	–	–	–	–	–
	5	AM	–	0.5	2.75	1.25	2.25	1.0	–	–	–	–
		SF	–	+	≧3.5	2.25	2.25	1.75	+	–	–	–
		Serum	–	NT	2.3	+	–	–	–	–	–	–
	6	AM	–	0.5	3.0	+	–	+	+	–	0.5	–
		SF	–	–	2.5	0.75	1.0	–	–	–	–	–
		Serum	–	NT	+	+	+	–	+	–	–	–
Cont.	7	AM	–	–	–	–	–	–	–	–	–	–
		SF	–	–	–	–	–	-	–	–	–	–
		Serum	–	–	–	–	–	-	–	–	–	–
	8	AM	–	–	–	–	–	-	–	–	–	–
		SF	–	–	–	–	–	-	–	–	–	–
		Serum	–	–	–	–	–	-	–	–	–	–
	9	AM	–	–	–	–	–	-	–	–	–	–
		SF	–	–	–	–	–	-	–	–	–	–
		Serum	–	–	–	–	–	-	–	–	–	–

a) AM: alveolar macrophage sample, SF:supernatant sample of broncho-alveolar lavage fluid.
b) No virus detected.
c) $LogTCID_{50}$/0.05 m*l*.
d) Positive for CPE in at least one of the wells inoculated.
e) Not tested.

Table 2. Antibody titers in sera collected from inoculated pigs against porcine reproductive and respiratory syndrome virus by indirect immunofluorescent assay

Group	Pig No.	Days after inoculation								
		0	7	14	21	28	35	42	49	56
Inoculated	1	<20	<20	80	160	320	1280	640	80	NT[a]
	2	<20	<20	160	160	320	640	160	160	NT
	3	<20	<20	40	80	320	640	320	80	NT
	4	<20	<20	40	80	160	320	80	80	80
	5	<20	<20	80	160	320	320	1280	640	40
	6	<20	<20	160	160	160	640	640	320	80
Cont.	7	<20	<20	<20	<20	<20	<20	<20	<20	<20
	8	<20	<20	<20	<20	<20	<20	<20	<20	<20
	9	<20	<20	<20	<20	<20	<20	<20	<20	<20

a) Not tested.

as described previously [10, 11]. The major advantage of using BALF sample collected from live pigs by broncho-alveolar lavage for a diagnostic sample is that the pig is not sacrificed and can be repeatedly examined. In the present study, lysate of AM or SF samples were used for virus isolation. Mengeling *et al*. [11] described that the sensitive method for isolating PRRS virus from AM was cocultivation of AM and MARC-145 cells. We showed that some PRRS

virus isolated from AM in Japan did not show CPE in MARC-145 cells [15]. Consequently, further investigations are required concerning the use of MARC-145 cells for cocultivation with AM.

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