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Interactions of Pseudorabies Virus With Swine Alveolar Macrophages: Effects of Virus Infection on Cell Functions

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In order to assess the effect of pseudorabies virus (PRV) infection on the function of swine alveolar macrophages (AM), lung lavage cells were cultured, infected with one of six strains of PRV, and various activities were measured. Activity measurement included viability, phagocytosis of yeast, phagosome-lysosome fusion, phagocytosis of opsonized particles, and superoxide release. AM were infected with 5×10^{-3} PFU/cell, and the comparative assessment of functions was performed at 18–20 h postinfection. Cell viability in PRV-infected cultures ranged from 79 to 94% of the viability in noninfected cultures. Phagocytosis of yeast was significantly reduced only in the AM cultures infected with the strain S-62. Phagosome-lysosome fusion was depressed in cultures infected with the strains S-62, 4892, 3816, and BUK. The phagocytosis of opsonized sheep red blood cells showed significant differences between noninfected and PRV-infected cultures in all cases except cultures infected with the strain PRV-C. The O_2 release after stimulation with opsonized zymosan was significantly reduced in all the PRV-infected cultures. The effect of PRV infection on AM functions that are related to the bacterial activity of such cells suggests that PRV-induced AM dysfunction might have a role in the increased susceptibility of PRV-infected pigs to bacterial pneumonia.

Key words: Aujeszky's disease virus, phagocytosis, O_2 metabolism

INTRODUCTION

The defense mechanisms of the lung against pathogenic bacteria may be divided in two categories: 1) mechanisms that physically remove bacterial material from the lung, and 2) mechanisms that destroy the viability of the organisms [13,21]. Comparative assessment of such mechanisms was performed using ^{32}P-radiolabeled bacterias [14]. Physical removal as measured by radioactive counts declined 14–20% by 4 h postinfection, whereas bacterial viability declined 80–90% by the same time. Another experiment [24] demonstrated that bacterial clearance from lung was significantly reduced in mice infected with Sendai virus even though the physical removal of particles was not affected. It was clear, therefore, that the bactericidal action of the lung predominated over the mechanical removal process in achieving clearance of inhaled bacteria. The bactericidal activity at the alveolar level is solely carried out by phagocytes, and it can be compromised by virus infections [6,21,22].

Chronic pneumonia in swine is a worldwide problem for the swine industry, and it is usually associated with proliferation of gram negative bacteria, bacteria commonly found in the pig respiratory tract [11,29]. Some viruses have been identified as primary pathogens, i.e., affecting the defense mechanisms and allowing bacteria to proliferate, such as influenza [4], hog cholera [34], and pseudorabies [5]. Pseudorabies virus (PRV), otherwise known as Aujeszky's disease virus, is primarily associated with reproductive failure and a high rate of mortality in suckling pigs [3], but in the last few years evidence has accumulated associating PRV in swine respiratory disease [1,39,40]. Most of the information is derived from clinical reports, but there is also a report on the production of pneumonia by experimental instillation of *Pasteurella multocida* in PRV-infected pigs. The development of pneumonia and the lesions in PRV-infected pigs was compared to that in control pigs that had not been infected with PRV; *P. multocida*-induced lesions were only observed in pigs that had been previously infected with PRV [9].

There is little information, however, on the type of impairment induced by PRV on the lower respiratory tract defense mechanisms. A recent study [19] showed

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that swine alveolar macrophages (AM) were highly permissive to PRV infection; virus-induced morphological changes and virus progeny titers were dependent upon virus input of infection and virus strain. AM cultures inoculated with 5×10^{-1} plaque-forming units (PFU)/cell of PRV strain S-62 or 4892 had >70% cell destruction by 24 h postinoculation. Progeny titers at 48 h postinoculation ranged from $10^{4.25}$ to $10^{7.5}$ $TCID_{50}/ml^{-1}$. In regard to in vivo infection, studies using experimentally infected pigs have shown that PRV can infect AM during the course of the disease [20].

The aim of this study was to determine if PRV infection has an effect on those macrophage functions that have a direct bearing on bactericidal activity, i.e., phagocytosis, phagosome-lysosome fusion, and oxidative metabolism.

MATERIALS AND METHODS

Alveolar Macrophages: Culture and Infection

AM were collected from 6- to 8-week-old pigs and cultured in vitro following procedures previously described [19]. The PRV strains used were all plaque purified, and all stocks were prepared by propagation in vero cells. The strains can be broadly divided into two groups: 1) field isolates—S-62, 4892, and 3816; and 2) laboratory-adapted (attenuated) strains—BUK, Bartha, and PRV-C. Previous studies showed that the cell destruction produced by virus replication was dependent on virus input [19]. In order to evaluate AM functional changes without a high proportion of destroyed cells, the multiplicity of infection (m.o.i.) employed was 5×10^{-3} PFU/cell. AM monolayers were inoculated with such a m.o.i. of one of six virus strains of PRV in Hanks' balanced salt solution for 1 h at 37°C, then washed with phosphate-buffered saline (PBS) to remove nonabsorbed virus. The proportion of viable cells at different times after infection was assessed by quantitation of neutral red uptake following the procedures described by Laude et al. [25].

Phagocytosis of Nonopsonized Particles and Phagosome-Lysosome Fusion

The phagocytosis of nonopsonized particles and the subsequent phagosome-lysosome fusion were evaluated using yeast particles [23]. Briefly, *Candida albicans* grown in Saboreaud dextrose agar plates was resuspended and washed repeatedly in PBS and then inactivated by heating at 95°C for 30 min. After inactivation the yeast solution was resuspended in PBS to a concentration of 10^8/ml. The infected and noninfected AM to be tested were cultured on 14-mm glass coverslips in 24-well microtiter plates. The culture medium was removed and 0.2 ml of a 1:2 dilution of yeast solution in PBS was added, followed by incubation at 37°C for 1 h. The monolayers were then washed twice with PBS, fixed with acetone:methanol (50:50) for 15 min, and stained with Giemsa stain. The number of cells showing intracellular yeast in 200 cells was recorded. The detection of phagosome-lysosome fusion was performed following procedures described by Hart and Young [16]. Briefly, acridine orange was added to cultures (1 μg/culture) 20 min before challenging with *Candida albicans*. After incubation with yeast solution (1 h 37°C), plates were washed twice, coverslips were wet-mounted onto glass slides, and cells were examined under incident ultraviolet light. Cells with fused phagosome-lysosomes had distinguishable orange intracytoplasmic granules. The number of cells showing "orange granules" in 200 cells was recorded.

Phagocytosis of Opsonized Particles

Fc-mediated phagocytosis was measured using ^{51}Cr-labeled opsonized sheep red blood cells (SRBC) following the procedures described by Plaeguer-Marshall et al. [35]. Five percent (v/v) SRBC were mixed with 1/10 (v/v) of hyperimmune rabbit anti-SRBC, incubated at 37°C for 1 h, and washed extensively with PBS. The amount of opsonized cells required for a 1% working solution was collected from the last pellet resuspended in 0.5 ml of PBS, and 100 μCi of $Na_2{}^{51}CrO_4$ was added. The cells were labeled by incubation at 37°C for 1 h with gentle agitation, washed twice, and 0.3 ml of a 1% solution was added to each 16-mm well containing AM. Phagocytosis was allowed to proceed for 1 h at 37°C followed by washing with PBS to remove nonphagocytized SRBC. Extracellular erythrocytes were lysed by hypotonic shock (0.5 ml of 0.2% NaCl/well, and 40 s later the same amount of 1.6% NaCl). After further washing, 1.0 ml of 1% Triton X-100 was added to each well. The total contents of each well were transferred to a tube, and the radioactivity was counted in a gamma counter. Each test was evaluated in triplicate.

Assessment of O_2 Release

PRV-infected or -noninfected AM cultured in siliconized tubes were pelleted by centrifugation at 600*g* × 10 min and resuspended in 0.2 ml of Hanks' solution. The O_2 release was measured following the procedures described by Nagahata et al. [31], with some modifications in order to use macrophages rather than polymorphonuclear cells. Both the nitroblue tetrazolium (NBT) concentration and the time of the assay were defined based on preliminary experiments carried out with noninfected cells. NBT reduction was clearly observed with concentrations of 0.05% NBT or higher. No significant difference was seen with concentrations between 0.1% and 0.2%. Thus, 0.1% NBT was used for all further analyses. The amount of formazan (final product of the NBT

reduction) produced after stimulation increased over time, reaching a plateau after 80 min. For all further analyses an incubation time of 90 min was chosen. The optimal procedure consisted of adding 50 μl of opsonized zymosan (10 mg/ml) and 0.2 ml of 0.1% NBT to infected or noninfected cells (2×10^6 cells/tube). The zymosan had been previously opsonized by incubation with normal pig serum for 1 h. After incubation at 37°C for 90 min, the reaction was stopped by the addition of 1 ml of 0.5 M HCl. The cells were then pelleted by centrifugation, washed again with 0.25 M HCl, resuspended in 2 ml of dimethylformamide, and heated for 15 min in a boiling water bath to extract the formazan. The color of the reaction was increased by adding 1 ml of 5 N NaOH. The solutions were thoroughly mixed and then separated by centrifugation at $500g \times 10$ min. The absorbance of the upper phase (formazan dissolved in dimethylformamide) was read at 710 nm using dimethylformamide as a blank. The results were expressed as optical density (O.D.) at 710 nm.

In order to examine a potential dose-effect relationship of the NBT reduction, an experiment was carried out using AM infected at m.o.i.s ranging from 5×10^{-5} to 5×10^{-2} with PRV strains 4892 and S-62. In this experiment the volume collected from the original tube was increased in the infected cells in order to have similar amounts of live cells in all samples.

Statistical Analysis

Each experiment was repeated 6 times, and the significance of the PRV-induced effect on each particular function of the AM was determined using an analysis of variance following the complete randomized block design [28].

RESULTS

Preliminary experiments carried out in order to determine the time course of PRV-induced effects on phagocytosis and phagosome fusion revealed that no effect was observed between 2 and 10 h postinfection, while from 40 h postinfection onward the effect could not be evaluated accurately because of the high proportion of detached cells. Therefore, the comparative assessment of activities was performed at 18–20 h postinfection.

The viability of PRV-infected AM was assessed at 18 h postinfection. The O.D. values in infected cultures were compared to the values obtained in noninfected cultures. The mean values were S-62, 79%; 4892, 82%; 3816, 80%; Bartha, 94%; BUK, 88%; PRV-C, 86%.

Phagocytosis of nonopsonized yeast particles was not affected by PRV infection as measured by the ratio of cells showing internalized particles over the total of cells, except in the case of cells infected with S-62 (Table 1). Phagosome-lysosome fusion was depressed by PRV infection in cultures infected with strains S-62, 4892, 3816, and BUK (Table 1).

TABLE 1. Effect of PRV Infection of Swine AM on the Phagocytosis of Nonopsonized Particles and Phagosome-Lysosome Fusion

Virus	Percent of cells with phagocytized yeast	Percent of cells positive to phagosome-lysosome fusion
None	96 ± 1	70 ± 8
S-62	78 ± 12*	55 ± 5*
4892	82 ± 6	46 ± 12**
3816	89 ± 5	54 ± 5*
BUK	86 ± 6	59 ± 6*
Bartha	88 ± 5	72 ± 6
PRV-C	93 ± 1	63 ± 6

*Significantly different at $P < .05$.
**Significantly different at $P < .025$.

TABLE 2. Effect of PRV Infection of Swine AM on the Phagocytosis of Opsonized Particles (Fc-Mediated Phagocytosis)

Virus	c.p.m. ± S.E.
None	2,809 ± 96
USDA	1,936 ± 45**
4892	1,849 ± 64***
3816	2,025 ± 62**
BUK	1,902 ± 81***
Bartha	2,120 ± 46*
PRV-C	2,320 ± 87

*Significantly different at $P < .05$.
**Significantly different at $P < .025$.
***Significantly different at $P < .01$.

Phagocytosis of opsonized particles (Fc-mediated phagocytosis) as measured by the internalization of opsonized SRBC was also negatively influenced by PRV infection, except in the case of one virus strain (Table 2). Experiments carried out using increasing concentrations of virus input showed that the Fc-mediated phagocytic activity was inversely related to the virus input. In AM cultures infected at a m.o.i. of 5×10^{-2} PFU/cell there was essentially no phagocytosis of SRBC (Fig. 1).

The metabolic activity measured by the amount of NBT reduced after stimulation with opsonized zymosan particles was severely depressed following PRV infection. This observation was valid for all six virus strains (Table 3). The experiments performed with increasing virus input showed that the decrease in metabolic activity was virus input dependent (Fig. 2).

DISCUSSION

Bacterial multiplication associated with virus infections in the respiratory tract is commonly regarded as a

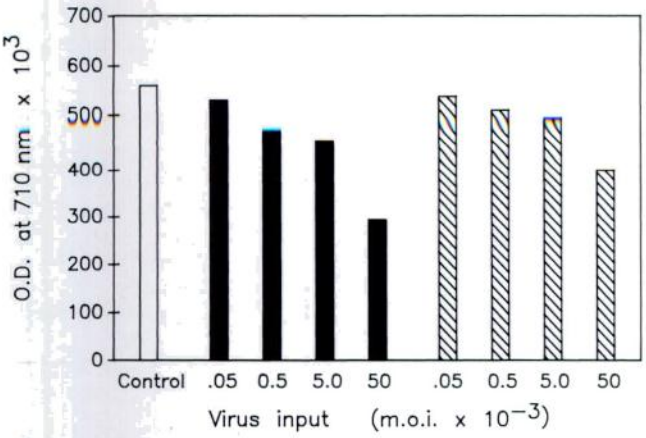

Fig. 1. Phagocytosis of ^{51}Cr-labeled antibody-coated sheep red blood cells by alveolar macrophages. □, Noninfected alveolar macrophages; ■, infected with PRV S-62; ▨, infected with PRV 4892.

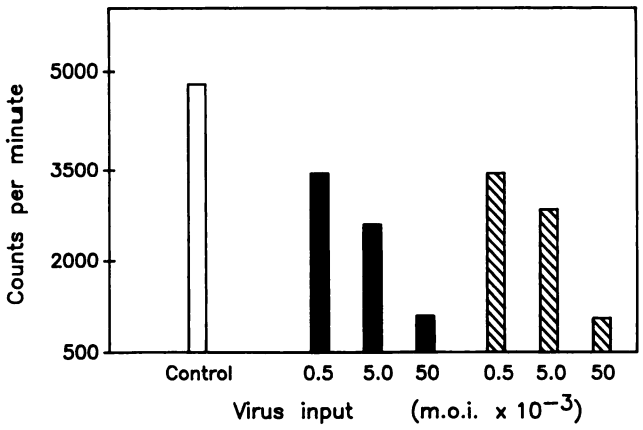

Fig. 2. Nitroblue tetrazolium reduction by alveolar macrophages after stimulation with opsonized zymosan. □, Noninfected alveolar macrophages; ■, infected with PRV, S-62; ▨, infected with PRV 4892.

TABLE 3. Effect of PRV Infection of Swine AM on NBT Reduction (O_2 Release) After Stimulation With Opsonized Zymosan

Virus	O.D. at 710 nm ± S.E.
None	.460 ± .43
USDA	.230 ± .50**
4892	.290 ± .61**
3816	.270 ± .44**
BUK	.240 ± .51**
Bartha	.380 ± .35*
PRV-C	.270 ± .39**

*Significantly different at $P < .05$.
**Significantly different at $P < .01$.

consequence of the virus-induced damage on bactericidal and phagocytic mechanisms of the lung [10,36]. Impairment of lung clearance by AM after virus infection is well documented [41]. The outcome of virus-AM interactions vary depending on the model of study used. Gortz et al. [12] found that herpes simplex virus type 1 (HSV-1) replicated to a higher titer in thiogylcollate-stimulated macrophages than in resident macrophages, whereas Howie et al. [18] reported that murine bone marrow macrophages were resistant to HSV-1. The experiments reported here are a follow-up of experiments that demonstrated the high permissiveness of swine AM to PRV infection [19]. It was clear that even at low virus input, PRV infection is able to result in impairment of AM functions. Decreased Fc-mediated phagocytosis has been reported to be affected with many other virus infections such as parainfluenza-3 (PI-3) virus in cattle [38] and other herpesviruses such as murine cytomegalovirus [37] and bovine herpes virus-1 [7]. In contrast, non-Fc-mediated phagocytosis was not significantly affected by PRV infection as measured by the internalization of yeast. The procedures used for the evaluation of each function were different, so while there is the possibility that phagocytosis of nonopsonized particles is not affected by infection, it is also possible that the counting of cells with internalized particles does not provide an accurate measurement of phagocytosis. This is because the total number of cells is affected by the detachment of cells, which is higher in the infected cultures. In a similar experiment carried out by Hesse and Toth [17] using PI-3 virus infection of bovine AM, a normalization equation was devised, which took into account the difference in total cells at counting. We realized during the preliminary experiments that a low number of cells remained attached to the plate at 24 h postinfection in cultures infected with m.o.i. of 5×10^{-2} or higher. Similarly to virus input, the detachment of cells increased with time after infection. Even though low virus input and a time postinfection that did not show high proportion of cells detaching from the plates were chosen, the total number of cells might have varied between PRV-infected and -noninfected cultures particularly in those infected with PRV field strains, known to replicate extensively in AM [19].

Phagosome-lysosome fusion was depressed in PRV-infected AM except in the case of cultures infected with strains Bartha or PRV-C. These two strains do not replicate in AM to the same extent as the field strains [19]. Phagosome-lysosome fusion failure has been associated with virulence mechanisms developed by some intracellular pathogens such as *Mycobacterium, bovis, lepraemurium,* or *avium* [8,26], and it has also been suggested as an impairment caused by virus infection [17,35].

The reduction of NBT has been used for the detection of super oxide anion (O_2^-) release in different kinds of phagocytic cells [33,43]. Oxygen release is a measurement of the respiratory burst [2]. The microbicidal efficiency of such activity has been clearly observed in the study of intracellular pathogens such as leishmania or toxoplasma [30,42]. Haidaris and Bonventre [15] reported that in cultured peritoneal mouse macrophages the development of leishmanicidal capacity was correlated with enhanced O_2 metabolism. The results presented here showed that PRV-infected AM were severely reduced in their ability to release O_2 upon stimulation with opsonized zymosan. Impairment was dose dependent and was exerted by all the PRV strains tested. There are two reports on the O_2 release of lung cells collected from experimentally infected animals. In both cases, one with cytomegalovirus and one with bovine herpes virus-1, the O_2 release was significantly affected [27,32]. It was interesting to note in our in vitro experiments that AM cultures infected with even a very low virus input (0.0005 PFU/cell) had decreased O_2 release (Fig. 2).

Clearly Fc-mediated phagocytosis and NBT reduction were drastically affected by PRV infection. The difference in activity as compared to the noninfected AM was significant at the $P < .01$ level in cultures infected with some strains, e.g., 4892, S-62, while the nonvirulent strains, Bartha and PRV-C, produced a less significant effect. It was previously reported that AM cultures infected with nonvirulent strains of PRV yielded progeny titers 2 to 3 log 10 units below the titers yielded by cultures infected with virulent strains [19]. Since such differences in progeny titers are not observed when PRV strains are propagated in fibroblastic or epithelial cell lines, it can be considered to be a cell-related function; in addition, the results presented here indicate that AM functions were not affected in a significant way by the infection of any of these two strains (PRV-C and Bartha). This inability to proliferate and induce macrophage impairment can be related to the lack of virulence of such strains.

The results of the experiments presented here leave no doubt that PRV infection leads to AM dysfunction even before cell death that is due to virus replication. Since the affected functions are relevant for bacterial clearance it is very likely that the effects of PRV or AM contribute to the enhanced susceptibility of PRV-infected pigs to bacterial pneumonia.

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