Veterinary Microbiology 231 (2019) 116–119

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Short communication

Administration of granulocyte-colony stimulating factor (G-CSF) to pigs results in a longer mean survival time after exposure to *Streptococcus suis*

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ARTICLE INFO

Keywords:
Granulocyte colony-stimulating factor
G-CSF
Streptococcus suis
Swine
Neutrophils

ABSTRACT

The use of immunomodulators is a promising alternative to the use of antibiotics for therapeutic, prophylactic, and metaphylactic use to prevent and combat infectious disease. Previously we demonstrated a replication-defective adenovirus vector that expresses porcine granulocyte colony-stimulating factor (G-CSF) elicited a sustained neutrophilia, lasting nearly 3 weeks, which may be beneficial to prevent bacterial diseases during times of peak incidence. In a pilot study using the vectored G-CSF with a Caesarian-derived, colostrum-deprived (CDCD) pig model of *Streptococcus suis* disease, only 1 of 4 pigs given G-CSF developed disease, while 3 of 4 non-treated pigs developed Streptococcal disease. In a subsequent study using a larger number of pigs, although there was no difference in overall survival, there was a longer mean survival time in G-CSF treated pigs. *S. suis* infection is more severe in CDCD pigs than conventionally raised pigs, consequently results in the field may be superior to the ones reported in this study. Although there were positive effects from the use of G-CSF in this study, further research is needed to determine if improved clinical outcomes could be achieved under field conditions and whether the use of G-CSF in pigs to induce a sustained increase in circulating neutrophil numbers may be useful as an adjunct to antibiotics to diminish the severity of Streptococcal disease, especially during times of stress and pathogen exposure such as post-weaning.

1. Introduction

Due to the increased pressure to reduce antibiotic usage in production animal medicine, alternatives to antibiotics for the treatment of bacterial infections need to be explored. Biotherapeutics such as immunomodulators are attractive candidates to stimulate or restore the ability of the immune system to combat infections, especially during times of stress and potential immune dysfunction. In particular, cytokines offer a specific method of action and should pose no food safety issues as they occur naturally in the animal. Granulocyte-colony stimulating factor (G-CSF) stimulates proliferation and release of neutrophils from the bone marrow. These professional phagocytes are critical to the innate control of many bacterial infections.

Previously we described that delivery of porcine G-CSF to swine through a replication-defective adenovirus vector resulted in elevated neutrophil counts for over two weeks (Loving et al., 2013). In addition, evaluation of the vectored-delivery of porcine G-CSF as a prophylactic to reduce *Salmonella* in pigs resulted in G-CSF-treated pigs shedding significantly less *Salmonella* in their feces and a significant reduction in tonsil colonization (Bearson et al., 2016).

Streptococcus suis is a bacterium that is an important and common cause of systemic disease such as meningitis, polyserositis, arthritis and septicemia in pigs, contributes to the porcine respiratory disease complex, and is also a zoonotic threat. Streptococcal disease has been reported to affect over 50% of the finishing herds, 75% of the breeding herds, and 95% of the nursery herds in the US (NAHMS 2012 survey). Strain variability and lack of cross-reactivity have made development of efficacious vaccines difficult, and thus there is significant antibiotic use to control this disease. Neutrophils are believed to be important for clearance of *S. suis in vivo* (Benga et al., 2008; Chabot-Roy et al., 2006), therefore, the purpose of this experiment was to determine if presence of increased circulating neutrophils induced by the administration of vectored G-CSF would decrease the incidence or severity of disease with *S. suis*.

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https://doi.org/10.1016/j.vetmic.2019.03.010
Received 17 September 2018; Received in revised form 8 March 2019; Accepted 10 March 2019

2. Materials and methods

2.1. Bacterial, viral vector, and sample culture

S. suis ISU1606 is a serotype 2, sequence type 1 strain that was isolated from a 6 week old pig from Iowa with signs of Streptococcal disease and was previously tested and shown to cause disease in Caesarian-derived, colostrum-deprived (CDCD) pigs (Hau et al., 2015). For inoculum, *S. suis* was grown on tryptic soy agar (TSA) containing 5% sheep blood (Becton, Dickinson and Co.) at 37 °C overnight, scraped from the plates and resuspended in phosphate buffered saline (PBS) to an optical density of 0.42 at A600, which corresponds to approximately 1×10^9 colony forming units (cfu)/ml. Replication deficient adenoviruses expressing porcine G-CSF (Ad5-G-CSF) (Loving et al., 2013) or without an inserted gene (Ad5-empty) were propagated in specialized AD-HEK-293 cells genetically altered to support replication. For inoculation, adenovirus isolates were purified and concentrated by double CsCl density gradients. Samples collected at necropsy were cultured on TSA containing 5% sheep blood at 37 °C overnight for growth of *S. suis* which was confirmed by colony morphology and species specific PCR.

2.2. Pig experiment 1

Eleven, 8-week-old CDCD pigs were divided into three groups. Pigs in group 1 (3 pigs – Ad5-G-CSF only) and group 2 (4 pigs – Ad5-G-CSF/*S. suis*) were given 1×10^{10} 50% tissue culture infective doses ($TCID_{50}$) of Ad5-G-CSF in 1 ml volume intramuscularly, while pigs in group 3 (4 pigs – *S. suis* only) were given 1×10^{10} $TCID_{50}$ of Ad5-empty in 1 ml volume intramuscularly. Two days later pigs in groups 2 and 3 were challenged with 2 ml containing 8.0×10^8 cfu/ml *S. suis* strain ISU1606, intranasally. On the day of adenovirus inoculation, the day of challenge with *S. suis*, and days 4 and 6 after *S. suis* challenge, a blood sample was collected from the pigs to determine their neutrophil counts and for culture for *S. suis*. After challenge with *S. suis,* pigs were observed for clinical signs of disease, including lameness, lethargy, and neurological symptoms. If presentation was severe (such as neurologic involvement, severe lameness, or depression that resulted in recumbency with reluctance to stand), the pig was euthanized. Pigs not showing signs of disease or only transitory or mild signs of disease were euthanized 14 days post challenge. At necropsy nasal and serosal swabs, joint fluid from the hock joint (or other affected joint), cerebrospinal fluid, and lung lavage were collected for culture.

2.3. Pig experiment 2

Nineteen, 12-week-old CDCD pigs were divided into 2 groups. Pigs in group 1 (10 pigs – Ad5-G-CSF/*S. suis*) were given 1×10^{10} $TCID_{50}$ of Ad5-G-CSF in 1 ml volume intramuscularly, while pigs in group 2 (9 pigs – *S. suis* only) were given 1×10^{10} $TCID_{50}$ of Ad5-empty in 1 ml volume intramuscularly. Three days later pigs in both groups were challenged with 2 ml containing 2.5×10^8 cfu/ml *S. suis* strain ISU1606, intranasally. On the day of adenovirus inoculation and the day of challenge with *S. suis* a blood sample was collected from the pigs to determine their neutrophil counts. After challenge with *S. suis*, pigs were observed for clinical signs of disease, including lameness, lethargy, and neurological symptoms. If presentation was severe (such as neurologic involvement, severe lameness, or depression that resulted in recumbency with reluctance to stand) the pig was euthanized. Pigs not showing signs of disease or only transitory or mild signs of disease were euthanized 14 days post challenge. At necropsy nasal and serosal swabs, joint fluid from the hock joint (or other affected joint), cerebrospinal fluid, lung lavage, and serum were collected for culture. Survival curves were compared statistically by the Mantel-Cox test and Gehan-Breslow-Wilcoxon test using GraphPad Prism 7.01.

2.4. Neutrophil counts

Peripheral blood neutrophil counts were performed *via* flow cytometry as described previously (Loving et al., 2013). Briefly, a 50-µl aliquot of anti-coagulated (EDTA) whole blood was added to a tube containing monoclonal antibody to porcine granulocytes (6D10, Serotech, USA) with appropriate secondary fluorochrome-labeled antibody. After a 20-min incubation, cells were fixed and red blood cells lysed with the addition of 1 ml FACS lyse (BD Biosciences, USA). Microbeads (Spherotech, USA) were added to the tube immediately prior to data acquisition on a flow cytometer (BD LSR II, Becton Dickinson, USA). A gate was drawn around the beads and events were collected on each parameter (neutrophil gate was based on forward and side scatter properties and antibody labeling) until the bead event number was 500. A ratio of total counts to bead counts was used to determine the number of neutrophils per microliter of blood.

3. Results and discussion

3.1. Pig experiment 1

Prior to giving the Ad5 vectors (day -2) the peripheral blood neutrophil counts for all pigs were within normal limits, with the mean for each group ranging from 2213 to 4490 neutrophils per µl of blood (Fig. 1). Two days after injection with the Ad5-G-CSF vector, on the day of challenge with *S. suis* (day 0), the mean neutrophil counts had risen significantly to a mean of approximately 50,000/µl of blood, and the neutrophil levels continued to rise to peak mean values over 100,000/µl, 6 days after Ad5-G-CSF injection (day 4 post challenge). Eight days after Ad5-G-CSF injection mean neutrophil levels were lower than peak levels but still elevated. There was no statistical difference in the mean neutrophil counts between the Ad5-G-CSF only and the Ad5-G-CSF/*S. suis* groups. Neutrophil counts were slightly higher for pigs in the *S. suis* only group on day 4 compared to the day of challenge likely in response to the *S. suis* infection, but the counts were significantly lower than those seen in the pigs given Ad5-G-CSF.

One pig from the Ad5-G-CSF/*S. suis* group was unable to rise the morning of day 2 after challenge with *S. suis* and was euthanized. *S. suis* was isolated from the serosal swab, joint fluid, cerebrospinal fluid, and lung lavage from this pig. On day 4 post challenge, *S. suis* was isolated from the blood cultures of two out of the four of the *S. suis* only pigs, but not from any of three surviving Ad5-G-CSF/*S. suis* pigs or from the three Ad5-G-CSF only pigs. Five days after challenge three of the four pigs in

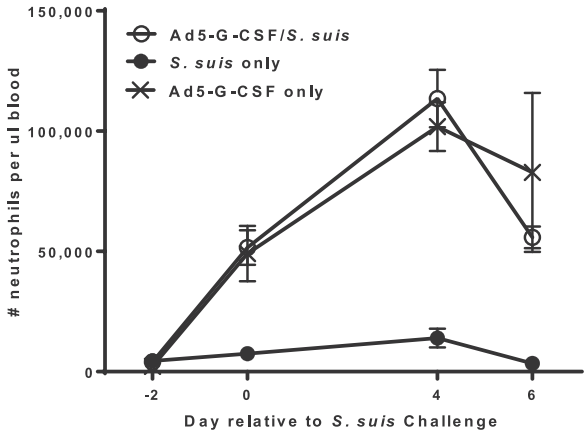

Fig. 1. Ad5-G-CSF induced a neutrophilia in pigs. Mean neutrophil counts in the peripheral blood after injection with adenovirus expressing G-CSF (Ad5-G-CSF only and Ad5-G-CSF/*S. suis*) or the empty adenovirus vector (*S. suis* only) on day -2 in experiment 1. Pigs in groups Ad5-G-CSF/*S. suis* and *S. suis* only were subsequently challenged with *S. suis* on day 0. Data are expressed as the mean ± SEM.

Table 1
Number of pigs from each group in pig experiment 2 from which *S. suis* was cultured from various anatomical sites.

Group	CSF	Serum	Serosa	Joint	Lung lavage
Ad5-G-CSF/*S. suis*	5/10	5/10	0/10	0/10	1/10
S. suis	9/9	9/9	4/9	1/9	0/9

CSF = cerebrospinal fluid.

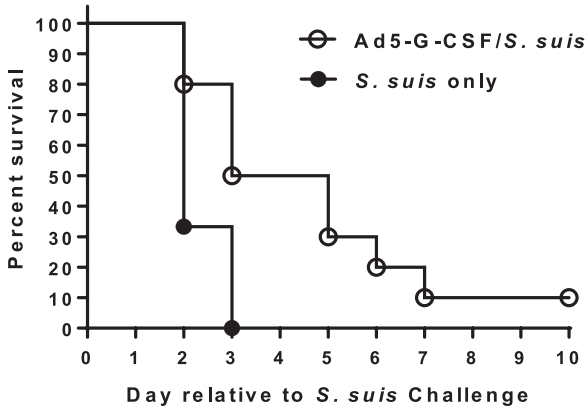

Fig. 2. Ad5-G-CSF induced neutrophilia resulted in a longer mean time to death after challenge with *S. suis*. Survival rates of pigs in experiment 2 that were either injected with Ad5-G-CSF and subsequently infected with *S. suis* three days later (Ad5-G-CSF/*S. suis*) or injected with the Ad5 empty vector and subsequently challenged with *S. suis* (*S. suis* only). Challenge was on day 0. The difference in survival was significant using Mantel-Cox test (P = 0.008) and Gehan-Breslow-Wilcoxon test (P = 0.0118).

the *S. suis* only group began showing lameness and neurologic signs and were euthanized. *S. suis* was isolated from the serosal swabs and cerebrospinal fluid from all three pigs and from the joint fluid from two and the lung lavage from one of the pigs. No *S. suis* was cultured from the blood taken on day 7 after *S. suis* challenge from any of the remaining pigs nor did any of these pigs develop clinical signs of *S. suis* infection. The three Ad5-G-CSF only pigs, three surviving Ad5-G-CSF/*S. suis* pigs, and one surviving *S. suis* only pigs were euthanized two weeks after challenge and *S. suis* was not isolated from any systemic site.

3.2. Pig experiment 2

With promising results from the first experiment, a second experiment was conducted with a larger number of pigs. Similar to the first experiment, neutrophil counts for all pigs were within normal limits prior to injection with the Ad5 vectors with mean counts of 2591 and 2052 neutrophils per μl of blood for the Ad5-G-CSF/*S. suis* and the *S. suis* only groups, respectively. Pigs given the Ad5-G-CSF had significantly higher neutrophil counts 3 days after administration on the day of challenge with *S. suis* (49,899 neutrophils per μl of blood) compared to the *S. suis* only group (2283 neutrophils per μl of blood). All nine pigs in the *S. suis* only group had to be euthanized due to neurologic signs of *S. suis* infection on days 2 and 3 post infection. *S. suis* was isolated from the cerebrospinal fluid and serum taken at necropsy from all 9 pigs as well as from some other sites in some of the pigs (Table 1). Although nine of the ten pigs given Ad5-G-CSF eventually had to be euthanized due to neurologic Streptococcal disease as well, there was a statistically significant longer mean time to development of clinical signs and euthanasia (Fig. 2). One pig in the Ad5-G-CSF group never developed signs of Streptococcal infection and was euthanized two weeks after challenge. There were also fewer *S. suis* isolations from these pigs (Table 1).

Results of these experiments demonstrated positive effects from prophylactic treatment with G-CSF on subsequent *S. suis* infection in swine. Neutrophils are believed to be important for clearance of *S. suis in vivo*. Neutrophils have been shown to be more proficient than mononuclear cells in uptake of capsulated *S. suis* (Benga et al., 2008). In agreement with our results, it has been shown by others that IL-1 treated pigs had less severe disease when subsequently challenged with *S. suis* which was associated with elevated neutrophil numbers and effector functions (Shi et al., 1994). Similarly, depletion of neutrophils was shown to exacerbate disease in mouse models of *S. pneumoniae* meningitis and pneumonia (Sun et al., 2007; Mildner et al., 2008; Too et al., 2016). However, excessive infiltration of neutrophils can sometimes result in disproportionate inflammation and enhancement of disease (Penaloza et al., 2015). Thus far we have observed no untoward effects or disease enhancement as a result of the increased circulation of neutrophils induced by giving G-CSF.

Caesarian-derived, colostrum-deprived pigs were used in these studies because they are very susceptible to *S. suis* infection and disease. *S. suis* disease can be difficult to reproduce consistently in conventionally reared pigs, probably due to a number of reasons such as maternally-acquired passive immunity, prior colonization with non-pathogenic strains, and possibly even differences in host microbiota. Opsonization has been shown to be important in phagocytosis of *S. suis* by neutrophils (Chabot-Roy et al., 2006). Thus, it is conceivable that G-CSF treatment may be more effective in the field, and additional research under conventional rearing conditions may be warranted. With alternatives to antibiotics being sought in production animal medicine, immunomodulators such as G-CSF may be a useful adjunct to antibiotics to mitigate the severity of infectious bacterial disease or reduce the quantity of antibiotics needed for treatment of disease, especially during times such as weaning when stress can lead to immune dysfunction and new pathogen exposure is occurring.

Acknowledgements

The authors would like to thank Steven Kellner, Zahra Olson, and Lilia Walther for their excellent technical assistance, and Jason Huegel, Aaron Hebeisen, Justin Miller and Keiko Sampson for their excellent animal care. This research was supported in part by appointments to the U.S. DEDA Agricultural Research Service Postdoctoral Research Program administered by the Oak Ridge Institute for Science and Education (ORISE) through an interagency agreement between the U.S. Department of Energy and the U.S. Department of Agriculture (KCE, KTM). ORISE is managed by ORAU under DOE contract number DE-AC05-06OR23100. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

References

Bearson, S.M., Bearson, B.L., Loving, C.L., Allen, H.K., Lee, I., Madson, D., Kehrli, M.E., 2016. Prophylactic administration of vector-encoded porcine granulocyte-colony stimulating factor reduces *Salmonella* shedding, tonsil colonization, and microbiota alterations of the gastrointestinal tract in *Salmonella*-challenged swine. Front. Vet. Sci. 3, 66. https://doi.org/10.3389/fvets.2016.00066.

Benga, L., Fulde, M., Neis, C., Goethe, R., Valentin-Weigand, P., 2008. Polysaccharide capsule and suilysin contribute to extracellular survival of *Streptococcus suis* co-cultivated with primary porcine phagocytes. Vet. Microbiol. 132, 211–219.

Chabot-Roy, G., Wilson, P., Segura, M., Lacouture, S., Gottschalk, M., 2006. Phagocytosis and killing of *Streptococcus suis* by porcine neutrophils. Microb. Pathogen. 41, 21–32.

Hau, S.J., Bayles, D.O., Alt, D.P., Brockmeier, S.L., Frana, T.S., Nicholson, T.L., 2015. Draft genome sequences of nine *Streptococcus suis* strains isolated in the United States. Genome Announc. 3 (6) e01301-15.

Loving, C.L., Kehrli, M.E., Brockmeier, S.L., Bayles, D.O., Michael, D.D., Schlink, S.N., Lager, K.M., 2013. Porcine granulocyte-colony stimulating factor (G-CSF) delivered via replication-defective adenovirus induces a sustained increase in circulating peripheral blood neutrophils. Biologicals 41, 368–376.

Mildner, A., Djukic, M., Garbe, D., Wellmer, A., Kuziel, W.A., Mack, M., Nau, R., Prinz, M., 2008. Ly-6G + CCR2- myeloid cells rather than Ly-6ChighCCR2 + monocytes are

required for the control of bacterial infection in the central nervous system. J. Immunol. 181, 2713–2722.
Penaloza, H.F., Nieto, P.A., Munoz-Durango, N., Salazar-Echegarai, J., Torres, J., Parga, M.J., Alvarez-Lobos, M., Riedel, C.A., Kalergis, A.M., Bueno, S.M., 2015. Interleukin-10 plays a key role in the modulation of neutrophils recruitment and lung inflammation during infection by *Streptococcus pneumoniae*. Immunol. 146, 100–112.
Shi, J., Goodband, R.D., Chengappa, M.M., Nelssen, J.L., Tokach, M.D., McVey, D.S., Blecha, F., 1994. Influence of interleukin-1 on neutrophil function and resistance to *Streptococcus suis* in neomatal pigs. J. Leukoc. Biol. 56, 88–94.
Sun, K., Salmon, S.L., Lotz, S.A., Metzger, D.W., 2007. Interleukin-12 promotes gamma interferon-dependent neutrophil recruitment in the lung and improves protection against respiratory *Streptococcus pneumoniae* infection. Infect. Immun. 75, 1196–1202.
Too, L.K., Mitchell, A.J., McGregor, I.S., Hunt, N.H., 2016. Antibody-induced neutrophil depletion prior to the onset of pneumococcal meningitis influences long-term neurological complications in mice. Brain Behav. Immun. 56, 68–83.