Effect of Immunostimulation by Detoxified *E. coli* Lipopolysaccharide Combined with Inactivated *Propionibacterium granulosum* Cells on Porcine Immunity

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**ABSTRACT.** The aim of this experiment was to evaluate the immunomodulating activities of inactivated Propionibacterium granulosum cell walls and *E. coli* lipopolysaccharide (PG/LPS) on porcine immunity. Piglets were intramuscularly administered PG/LPS (1 ml/10 kg body weight) once or twice. The function of natural killer cells, lymphocytes and neutrophils and the adjuvant effect on antibody induction by attenuated classical swine fever virus (CSFV) and inactivated Mycoplasma hyopneumoniae vaccination were evaluated. The results showed that the cytotoxicity of natural killer cells and proliferation of lymphocytes in response to mitogen stimulation were significantly enhanced (*P*<0.05) in those pigs receiving PG/LPS injection compared with the controls. However, there was no significant effect on the phagocytic activity of neutrophils (*P*=0.05). PG/LPS also displayed adjuvant effects with CSFV and Mycoplasma hyopneumoniae vaccines. Moreover, pigs receiving two injections of PG/LPS showed a 20.8% growth enhancement compared with untreated pigs. Thus, PG/LPS caused positive immunoregulation of porcine innate immune system effectors, non-specific activation of lymphocytes and antibody production.

**KEY WORDS:** adjuvant effect, immunomodulation, leukocyte function, lipopolysaccharide, Propionibacterium granulosum.

Porcine respiratory disease complex (PRDC) and porcine circovirus type 2 (PCV2) associated diseases (PCVAD) have become the major disease concern in most pig farms in the world. The contributing factors of PRDC and PCVAD are very complicated and may involve multiple pathogens, including PCV2, porcine reproductive and respiratory syndrome virus (PRRSV), influenza virus and Mycoplasma (*M.*) hyopneumoniae, as well as various stressors from the farm managerial system and pig’s environment [2, 15, 28]. Additionally, endemic classical swine fever (CSF) is a disease of particular concern in Asia, which complicates PRDC/PCVAD and makes the disease more severe than in other regions. With the decay of maternal protection, weaning pigs are at risk of developing various infections, and if CSF virus (CSFV) is endemic in pig herds, it would increase the severity of PRDC/PCVAD (unpublished data). The increased pathogen load and decreased natural immune activity play a critical role in development of PRDC [2]. Therefore, to try to solve the PRDC problem and prevent endemic CSF, strategies including vaccinations against CSFV and *M*. hyopneumoniae, antibiotic treatment and improvement of managerial and environmental conditions have been applied [34]. However, enhancement of natural immunity through administration of immunomodulators and nutrients is becoming very important as measures against infections, particular in the face of concerns of antibiotic abuse.

Immunomodulators can enhance innate immunity against various infections and reduce severity during infection. Oral administration of a bacterial immunomodulator intensifies the lymphocyte traffic from the intestinal lamina propria and Peyer’s patches to the lung, which is suggestive of enhanced host defense in the respiratory track [32]. Propionibacteria are anaerobic, gram-positive bacilli considered to have low pathogenicity to humans, although they have been implicated in a wide range of infections including endocarditis, endophthalmitis, osteomyelitis and central nerve system infection [4, 5]. Some strains of propionibacteria display immunostimulatory potency that can stimulate spleen enlargement and reduce tumor metastatic activity [17, 20]. Lipopolysaccharide (LPS) is a constituent of the outer membrane of gram-negative bacteria. Depending on dosage, it causes a wide range of physiological responses, including inflammation and stimulation of non-specific antibody secretion and differentiation of B-lymphocytes [18, 19, 21]. The combination of inactivated Propionibacterium granulosum (PG) cells and detoxified LPS from *E. coli* (PG/LPS) has displayed a broad range of immunostimulatory activities against bacterial and viral infections [11, 12] and enhancement of the immune response to cholera toxin [13]. *In vitro*, PG/LPS combination stimulates alveolar macrophages to release interleukin-1 (IL-1), IL-6 and IL-12 and peripheral blood mononuclear cells (PBMC) to release IL-1, IL-2, IL-4, IL-6, IL-12 and IFN-γ, which enhance humoral and cellular immune responses [29]. The immunostimulating effect of PG/LPS on cytokine production of porcine alveolar macrophages is much higher than...
administration of LPS alone [29]. PG/LPS also shows a marked immunostimulant effect on the increase in the number of antibody producing cells in a mouse model [14]. Therefore, it is possible that administration of PG/LPS could indirectly promote the activity of pulmonary alveolar macrophages, PBMCs, mucosal immunity and antibody production and also increase host resistance against respiratory and digestive tract infections. Reported data have indicated that PG/LPS is capable of reducing the number of antibiotic treatments given to pigs infected with swine dysentery [11] and swine enzootic pneumonia [12]. It also displays a good adjuvant effect when used in conjunction with pseudorabies vaccine [29]. These results indicate that PG/LPS is capable of non-specifically activating the immune system of the host and so elevate resistance to viral and bacterial infections.

Based on field results obtained thus far, PG/LPS possesses positive modulating effects on disease resistance, improving treatment and the antigenicity of vaccines. However, the effect of PG/LPS administration on leukocyte functions in swine has not been well addressed. Here, we report that PG/LPS positively regulated porcine NK cell cytotoxicity and lymphocyte activation and caused an adjuvant effect on M. hyopneumoniae and CSF vaccination, which may have potential application in the field for reduction of the levels of serious porcine respiratory diseases.

MATERIALS AND METHODS

**Propionibacterium granulosum with lipopolysaccharides (PG/LPS):** A commercial product, Inmodulen®, containing 25 mg of inactivated cells of Propionibacterium granulosum and 2 mg of lipopolysaccharides from E. coli in 100 ml aqueous vehicle was kindly supplied by Laboratorios Calier, S.A., Barcelona.

**Experimental pigs:** Experimental pigs were purchased from a conventional pig farm that had a history of good performance in the last three years. After weaning, the experimental pigs were raised in an isolation unit. At 6 weeks old, the pigs were divided into 3 groups (groups A, B and C), with 10 pigs in each group. Groups B and C received 1 ml of PG/LPS and a dose of M. hyopneumoniae vaccine (RespiSure®, Pfizer) intramuscularly in the right neck at the same site and a dose of the CSF vaccine (Lapinization-Philippine-Coronel virus, LPC) in the left neck. Group A received the same vaccination treatment as group B and 1 ml of normal saline solution as a placebo. Two weeks later, group C received an additional 1 ml of PG/LPS intramuscularly, whilst groups A and B received normal saline solution. Blood samples were taken every 1 or 2 weeks for leukocyte functional assays and CSFV and M. hyopneumoniae antibody detection.

The experiments in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of National Chung Hsing University (IACUC approval no. 95–810).

**Antibody detection:** The commercial CHEKIT® CSF Sero ELISA kit (Dr. Bommeli AG), a blocking ELISA, was used to detect CSF E2 antibody. The assay followed the manufacturer’s recommended procedures. The ELISA value was expressed as a percentage of the inhibition value (%) and calculated as Value (%)=[(ODneg – ODsample)/(ODneg – ODpos)] × 100%. Only values ≥ 60% were considered positive. M. hyopneumoniae antibody was detected using Herdcheck® ELISA kits (IDEXX Laboratories, Inc.). The assay followed the manufacturer’s recommended procedures. The ELISA value was expressed as a sample/positive ratio calculated as S/ (ODsample – ODneg)/(ODpos – ODneg). A sample was considered positive if the S/P ratio was ≥ 0.4.

**Leukocyte preparation:** Blood samples were drawn from the jugular vein into heparinized tubes. Leukocytes were first counted with an electronic haematology counter (Sysmex F-800). To obtain peripheral blood mononuclear cells (PBMCs), heparinized blood was sedimented with phosphate-buffered saline (PBS) containing 2% dextran (2:1) and then separated by a Ficoll-Hypaque (Pharmacia Biotech) gradient as previously described [16]. PBMCs were counted, and viability was determined by trypan blue exclusion.

**Cytotoxic activity of NK cells:** The assay was performed using a time-resolved fluorometer (TRF) system as previously described [16]. Briefly, the target cells, a human erythromyelocytic leukemia cell line (K562) growing in logarithmic phase, were harvested and labeled with 2 μl of enhancing ligand bis(acetoxymethyl) 2,2': 6', 2'-terpyridine-6,6'-dicarboxylate (BATDA, Wallac Labelling Service, Perkin Elmer) according to the manufacturer’s instructions. Effector cells were obtained from PBMCs after depletion of adherent cells in a culture flask for 30 min. The effector to target ratio was set at 100:1, and the mixture was incubated at 37°C in a 5% CO2 incubator for 4 hr. Cytotoxic activity was calculated by the release of the fluorescent dye that had been chelated with europium (EuTDA) from dead target cells. The percent specific release of fluorescence of EuTDA was measured in a time-resolved fluorometer (1234 Delfia, Wallac, Turku, Finland) and calculated as [(experimental release – spontaneous release)/ (maximum release – spontaneous release)] × 100%. The counts of spontaneous release ranged between 18% and 25% of the maximum label.

**Phagocytic activity of neutrophils:** The phagocytic activity of neutrophils used a whole blood method and was analyzed by flow cytometry as previously described [24]. Briefly, a stock of FITC (fluorescein isothiocyanate)–labelled Salmonella Typhimurium (ST; ATCC 13311) stored in 20% glycerol (8 × 108 cfu/ml) was used as the target. The leukocyte to bacteria ratio was set at 1:50 in a final volume of 100 μl of whole blood dependent on the leuko-
cyte number. Phagocytosis was performed at 37°C for 30 min. RBCs were treated with lysis buffer (distilled water containing 0.899% ammonium chloride, 0.1% potassium hydrogen carbonate and 0.0037% disodium EDTA), washed in chilled fluorescence-activated cell sorting (FACS) washing buffer (PBS containing 0.1% [w/v] bovine serum albumin and 0.01% [w/v] sodium azide), fixed in 1% paraformaldehyde in PBS and then stained with monoclonal antibody (74–22–15, ATCC) to porcine neutrophils followed by goat anti-mouse IgG-phycoerythrin (PE) conjugate, F(ab')2 (1:100; Sigma Chemical Co.). Ten thousand PE-positive cells (neutrophils) were collected by flow cytometer (Becton Dickinson Immunocytometry Systems; BDIS) using the CellQuest software (BDIS). The average phagocytic activities of phagocytes were determined by fluorescence intensity in the FITC color (FL-1) channel of 74–22–15 positive cells.

Lymphocyte proliferation assay: PBMCs were adjusted to a concentration of 2 × 10^6 cells/ml, and 100 μl of cell suspensions of PBMCs was plated in quadruplicate onto 96-well flat bottom microplates. One hundred microliters of concanavalin A (Con A, final concentration of 2 μg/ml per well) or medium alone (control) was added to each well and incubated at 37°C in 5% CO2 for 72 hr. After 54 hr of incubation, 0.5 μCi of tritiated thymidine (Amersham Life Science) was added to the cultures, and the cells were further incubated for 18 hr. Cells were harvested onto glass-fiber filter paper, and the radioactivity was counted using a liquid scintillation spectrometer (Beckman liquid scintillation system model LS8000). Data were expressed as a stimulation index (SI) whereby thymidine uptake (in counts per minute, CPM) of the stimulated cells was divided by the counts (CPM) of the unstimulated cells (control).

Statistical analysis: Statistical analyses of the data were calculated using analysis of variance (ANOVA) and Duncan’s multiple range tests, with P values of ≤ 0.05 taken as indicating significant differences.

RESULTS

Effect of PG/LPS on the cytotoxicity activity of natural killer cells: The cytotoxic activity of natural killer cells of pigs in group B receiving one PG/LPS injection was 27.6% on the 7th day post injection, and this was higher (45.4% relative increase) than the level of control, group A (15.1%, P<0.05; Fig. 1). On the 14th day post PG/LPS injection, the treated group exhibited a cytotoxic activity of 28.5%, which was still higher (29.4% relative increase) than the level of the control, group A (20.2%), although the difference was not significant (P>0.05). The cytotoxic activity of the treated group (group B) on days 7 and 14 was not significantly different (P>0.05).

Effect of PG/LPS on the phagocytic activity of neutrophils: All samples showed phagocytic activity by neutrophils for Salmonella. However, there were no statistically significant differences at days 7 (2,606 ± 762) and 14 (2,860 ± 332) in the PG/LPS treated group (group B) compared with the control group (2,458 ± 582 and 2,921 ± 552 respectively, P>0.05; Fig. 2).

Effect of PG/LPS on lymphoproliferation: The effect of PG/LPS on lymphocyte activation was assayed by lymphoproliferation to Con A stimulation. The stimulation indices at 7 and 14 days post injection in the PG/LPS treated group (group B) were 430.9 ± 582 and 2,921 ± 172, respectively, which were greater than the levels of the control, group A (233.0 ± 33.7 and 155.4 ± 46.7, P<0.05; Fig. 3).

Adjuvant effect of PG/LPS on CSF and M. hyopneumoniae vaccination: The immune response to CSFV vaccination was detected by CSF Ab ELISA (Fig. 4A). All pigs received the LPC vaccine at 6 weeks old, and antibody titers were monitored every 1 or 2 weeks. All pigs showed...
marked seroconversion on week 11 and an increased ELISA value on week 13. The ELISA value of CSF antibody in the pigs receiving PG/LPS on week 13 was significantly higher than the value of the control group \( (P<0.05) \), whereas there was no significant difference between pigs receiving 1 and 2 injections of PG/LPS \( (P>0.05) \). All experimental pigs were also vaccinated with \( \text{M. hyopneumoniae} \) vaccine at 6 weeks old, and anti-\( \text{M. hyopneumoniae} \) antibodies were detected by \( \text{M. hyopneumoniae} \) ELISA (Fig. 4B). All the experimental pigs were \( \text{M. hyopneumoniae} \) antibody negative at 6 weeks old. After vaccination, pigs gradually developed antibodies between 2 and 5 weeks later. At 7 weeks post vaccination, the seroconversion rate to \( \text{M. hyopneumoniae} \) vaccine in the pigs that received one and two injections of PG/LPS was 89% (mean S/P value=1.07) and 100% (mean S/P value=1.57), respectively, which were both significantly higher \( (P<0.05) \) than the 30% frequency (mean S/P value = 0.42) of the control group. Moreover, the seroconversion rate of the group receiving 2 injections of PG/LPS was also significantly \( (P<0.05) \) higher than that of the group that received one injection. At the same time, some control pigs that did not receive the \( \text{M. hyopneumoniae} \) vaccine were negative for \( \text{M. hyopneumoniae} \) antibody (0% positive rate) at 13 weeks of age (data not shown).

**Effect of PG/LPS on pig growth performance:** The average increase in body weight from day 0 to day 49 of the experiment was compared. The group receiving 2 injections of PG/LPS had a 20.8% increase in average body weight compared with the control group. There were no significant difference in body weight gain between the group receiving one injection of PG/LPS and the control group (Fig. 5).

**Fig. 3.** The effect of PG/LPS on lymphoproliferation. The mean ± SD of the group stimulation index to Con A is shown for group A (control) and group B (1 injection of PG/LPS) on 7 and 14 dpi. An asterisk (*) indicates significant differences \( (P<0.05) \) compared with the controls by ANOVA.

**Fig. 4.** The antibody responses of pigs after PG/LPS injection and CSFV or \( \text{Mycoplasma hyopneumoniae} \) (MP) vaccination. The mean anti-CSF inhibition value ± SD (A) and mean S/P ratio of MP antibody ± SD (B) of each group are shown against the time post inoculation. M: mean value of each group at week 13. The letter a denotes significant differences compared with b and c by Duncan’s multiple range test \( (P<0.05) \). The letter b indicates significant differences compared with a and c \( (P<0.05) \).

**Fig. 5.** Effect of PG/LPS on the growth performance of pigs. The mean ± SD of each group’s increase in body weight from day 0 to 49 days after the start of the experiment is shown. There was a 20.8% relative increase in body weight gain in group B (2 injections of PG/LPS) compared with the control group (group A).
DISCUSSION

The innate immune system is the first line of defense against infections mainly mediated by phagocytic cells, NK and dendritic cells. Immunomodulants such as PG/LPS can stimulate proinflammatory cytokine production and elevate the defense activity. After administration, PG/LPS may induce a serial signal activation; the PG cell wall may be recognized by C-type lectin receptor and induce inflammatory cytokine production [3]. Toll-like receptors (TLR) recognize diverse pathogen patterns and shape adaptive immune responses [22]. Therefore, the synergistic effect of PG/LPS may amplify the protective immunity against infections. As the LPS used in this study had been detoxified, there was no side effect noted after administration in this experiment.

Natural killer cells play an important role in innate immunity, helping to eliminate many intracellular pathogens. The activity of NK cells against pathogens includes destruction of infected cells and secretion of IFN-γ, which regulates the early adaptive immune response and is critical for activation of macrophages [8, 36]. Therefore, poor NK cell function is correlated with susceptibility to viral and other microbial infections [1]. Here, the significant enhancement of cytotoxic activity of NK cells after administration of PG/LPS was only noted in the first week of this experiment. This result is similar to a previous study showing that PG/LPS stimulates PBMCs to produce cytokines including IL-2 and IL-12 [29], which strengthen the function of NK cells. Nevertheless, it is still unclear how long this effect can be maintained. After a temporary stress, the activity of NK cells rises due to internal hormone-associated regulation [7, 16], but this factor would have existed in both the PG/LPS-treated and control groups in our study. Therefore, any difference between groups should have derived from the biological activity of PG/LPS. Samples from the second week after administration of PG/LPS showed that the cytotoxic activity of NK cells in the control group was also elevated such that there was no difference compared with the experimental groups, although the experimental groups showed the same level of activity in the first week’s samples. It may well be that, with larger group sizes, the cytotoxic activity of NK cells after PG/LPS-treatment may be found to be increased for more than 2 weeks post inoculation. However, it is also possible that the increase seen in the control group values at 2 weeks post-inoculation were a reflection of activation caused by the vaccinations.

Propionibacteria can significantly enhance both phagocytosis and intracellular digestion of macrophages in a mouse model [31]. However, no significant difference in the phagocytic activity of neutrophils was detected on days 7 and 14 after PG/LPS injection in this study. We infer that this product has no long-term enhancement effect on the phagocytic activity of neutrophils. This is supported by work using intravenous administration of LPS in pigs, which showed that neutrophil phagocytosis was increased for 6 hr, but returned to normal activity by 24 and 48 hr [27]. Thus, the affect on neutrophil phagocytosis may be more appropriately assayed within a much shorter time period. Additionally, it has been reported that pigs show a large individual variation in neutrophil phagocytic activity [9] that may affect the group size needed to show significantly different activity between treatments.

Enhanced antibody secretion was observed at 7 and 14 days post PG/LPS injection with vaccines of inactivated M. hyopneumonia and a live CSFV vaccine, respectively. A similar adjuvant effect was noted in a previous report using pigs immunized with live Aujeszky’s vaccine [29]. The adjuvant effect of PG/LPS may be associated with some cytokine secretion, such as IL-4, IL-6 or IFN-γ secretion from activating PBMCs, or with direct stimulation of B cells [28]. An increase in the number of antibody producing cells has been reported in mice after PG/LPS injection [14]. In our study, significant enhancement of lymphoproliferation was noted in pigs in the first and second week after PG/LPS injection. Activating T lymphocytes produces a cocktail of cytokines that play a very important role in immunomodulation. IL-2 and IFN-γ are two of the most important cytokines in immunoregulation of cell-mediated immunity [23, 26, 30] including the activation of NK cells and macrophages [23, 33]. Therefore, whether PG/LPS administration can enhance the activation and IFN-γ production of T lymphocytes is critical for immunomodulation. However, the results still imply that the mild immunomodulating effects on lymphocytes could have been brought about by the lymphoproliferation that it induced. Our results agree with a previous report that propionibacteria can significantly increase lymphocyte proliferation in response to PHA-stimulation in a mouse model [31].

In pig production, the average body weight gain of young pigs is often used as an important parameter for evaluation of growth performance. Here, the group treated twice with PG/LPS showed a 20.8% relative increase in weight gain as compared with the controls. This suggests that there is an improvement in the health of pigs that allows for more efficient growth. A similar improvement of growth performance through the positive regulation of host natural immunity against pathogen infections has been reported in chickens and pigs fed sugar cane extracts [10, 25]. The improvement in growth performance we observed is probably a non-specific effect of increased immunity to multiple pathogens and is probably not restricted to CSFV and Mycoplasma. Multiple infectious agents have been identified as potential risk factors for pigs in Taiwan. Reports have demonstrated that PRRS and PCV2 have been widespread epidemic diseases at pig farms in most areas of Taiwan [6, 35]. Furthermore, a recent study reported that the most frequently identified pathogens from 136 PRDC cases in Taiwan are PCV2, Pasteurella multocida, Mycoplasma hyopneumoniae, Salmonella enterica serotype Cholerae-suis, PRRSV and Actinobacillus pleuropneumoniae. Other pathogens such as Streptococcus suis and swine influenza virus are less frequently identified [37].
Taken together, PG/LPS had a positive immunoregulation effect on porcine innate immunity (NK activity) and adaptive immune response that may contribute to the restriction of viral infection. This was consistent with increased growth enhancement in pigs treated with PG/LPS. PG/LPS possessed good adjuvant effects, enhancing immune responses to both inactivated *M. hyopneumoniae* vaccine and attenuated CSFV vaccines. It could be administered concomitantly in the field with vaccines, thus giving both non-specific and specific immune effects to enhance porcine resistance to infection.

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