Effects of Sugar Cane Extract on Pseudorabies Virus Challenge of Pigs

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(Received 5 August 2005/Accepted 24 October 2005)

ABSTRACT. This experiment aimed to evaluate the efficacy of sugar cane extract (SCE) on the modulation of porcine immunity against pseudorabies virus (PrV) infection. Twelve-week-old experimental pigs were fed with SCE (500 mg/kg of body weight per day) for 3 days and challenged with PrV (2 × 10^5 TCID50) on the second day. Pigs that were only challenged with PrV and without SCE-treatment served as controls. The leukocyte functional assays were performed on the 7th and 14th day post-PrV challenge. Our results showed a significant enhancement (P<0.05) of natural killer cytotoxicity, lymphocyte proliferation, phagocytic function of monocytes, and interferon-gamma (IFN-γ) production of CD4+ and γδ T cells in the SCE-treated pigs compared with the controls. In addition, SCE administration reduced the severity of clinical signs and brain lesion in the course of disease in PrV-challenged pigs. SCE-treated pigs showed a 12% growth enhancement compared with untreated controls. SCE administration had an immunostimulating effect on porcine immunity that may subsequently enhance protective activities against PrV infection which may be extensively applied in field for the prevention of infections.

KEY WORDS: immunomodulation, interferon-gamma, leukocyte function, pseudorabies, sugar cane extract.

In recent years, porcine respiratory disease complex (PRDC) has become the major problem in most intensive pig farms causing massive economic loss. The causes of PRDC involve a complicated synergistic interaction between host immunocompetence, pathogens, and various stressors arising from the environment and management [28]. Pseudorabies virus (PrV) has been recognized as one of the most important primary pathogens for PRDC in fattening pigs [5, 13, 28] and antibiotics are commonly used to prevent secondary bacterial infections. As the abuse of antibiotics in veterinary medicine has been of particular concern from the viewpoint of public health, besides vaccination, major strategies against PRDC are to improve the managerial systems and environmental conditions and to modulate host immunocompetence.

Sugar cane extract (SCE) is a natural product, which has displayed a wide range of biological effects including immunostimulation [11], anti-inflammatory activity [19], activity as a vaccine adjuvant [10], and anti-stress effects [4]. Studies on chickens indicate that SCE acts as an adjuvant and has a protective effect against Eimeria tenella infection [9]. Other effects of SCE including antibacterial activities [1, 25], resistance to cold stress, and superoxide anion scavenging activity [26] have been reported. These results suggest that SCE can activate and enhance host natural immunity against infections. Our recent report also indicates that SCE can up-modulate porcine immunity, particularly in the enhancement of natural killer cytotoxicity which plays an important role in early viral infections [21]. Therefore, we are interested in elucidating the immunomodulatory function of SCE against viral infections, particularly in PrV infection, which may be beneficial to control or reduce the effect of PRDC. In this experiment, we showed that functional activities of leukocytes in pigs were positively modulated by SCE, thus reducing the negative effect caused by PrV infection.

MATERIALS AND METHODS

Experimental pigs and housing: Male, mixed-breed (Landrace × Duroc) pigs were purchased from a local commercial swine farm in which no anti-PrV gE antibody presented in finishers in the last two years. After weanling, piglets were raised in an experimentally controlled house. No prophylactic antibiotic treatment was administered during the test period. The pigs were housed in raised pens with adequate ventilation. The pigs in each pen could move freely and had easy access to food and water. They were given ad libitum access to a nutritionally balanced diet containing corn-soybean meal plus vitamins and minerals.

The use of pigs in this study was approved by the Ethical Committee of the Faculty of Veterinary Medicine, National Chung-Hsing University (approval number: 93–023).

Sugarcane juice (SCE): SCE product is kindly provided by Shin Mitsui Sugar Co. Ltd., Japan. The product contained 20% of extract which was prepared from sugar cane juice (Saccharum officinarum L.) by chromatographic separation on an ion exchange column and absorbed with
oilcake of rice bran for oral administration. The final product consists of crude protein (19.8%), crude fat (4.8%), crude fiber (7.5%), ash (18.8%) and nitrogen-free extracts (49.1%) [11]. The SCE product was administered at a dose of 500 mg/kg body weight per day in this study.

*Pseudorabies virus (PrV):* PrV (TNL strain) was kindly provided by the Provincial Research Institute of Animal Health of Taiwan, Republic of China. Viruses were propagated in the rabbit kidney cell line (RK-13) in Dulbecco’s modified Eagles medium supplemented with 2 mM L-glutamine, 100 µg ampicillin per mL, and 2% fetal bovine serum.

**Monoclonal antibodies (Mabs):** Mabs to porcine CD4 (74–12–4) and CD8 (76–2–11) were obtained from ATCC. Mabs to porcine CD4 (76–2–11) and sheep anti-mouse IgG, F(ab’)-FITC conjugate used as the secondary antibody were purchased from Sigma Chemical Co. The mouse anti-porcine IFN-γ PE (Phycoerythrin) conjugated antibody was purchased from Pharmingen/Becton Dickinson.

**Reagents:** Phorbol 12-Myristate 13 Acetate (PMA), iodiumycin, and brefeldin A (BFA) from ATCC. They were used to activate expression and to inhibit transport of intracellular cytokine, respectively. The FACS washing buffer (PBS containing 0.1% [w/v] bovine serum albumin and 0.01% [w/v] sodium azide), fixation buffer (PBS containing 4% paraformaldehyde), permeabilization buffer (PBS containing 0.1% saponin, Sigma), and lysis buffer (distilled water containing 0.899% ammonium chloride, 0.1% potassium hydrogen carbonate and 0.0037% disodium EDTA) were used for cell washing, fixation, permeabilization, and RBC lysis, respectively.

**Grouping and virus challenge:** The experimental pigs (12-week-old) were divided into two groups: control (untreated) group (N=6) - PrV inoculation without SCE administration; and SCE-treated group (N=6) - PrV inoculation with SCE administration. A dose (500 mg/kg of body weight per day) of SCE was supplemented in feed for one hundred microliters of concanavalin A (Con A, 2 µg/mL) were added to each well and plates were incubated at 37°C for 24 hr. After 54 hr incubation, 0.5 µCi of tritiated thymidine (Amersham Life sciences) was added to the cultures and cells were further incubated for 18 hr. Cells were harvested onto glass-fiber filter paper and 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.

**Lymphocyte proliferation assay:** PBMCs were adjusted to a concentration of 2 × 10^6 cells/mL and 100 µL were plated in quadruplicate into 96-well flat bottom microplate. One hundred microliters of concanavalin A (Con A, 2 µg/mL) or medium alone (control) were added to each well and plates were incubated at 37°C, 5% CO₂ for 72 hr. After 54 hr incubation, 0.5 µCi of tritiated thymidine (Amersham Life science) was added to the cultures and cells were further incubated for 18 hr. Cells were harvested onto glass-fiber filter paper and 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.

**Detection of intracellular interferon-gamma (IFN-γ):** Measurement of intracellular IFN-γ expression in T cell subsets was assayed by flow cytometry by a modified method from that previously described [24]. Briefly, PBMCs (2 × 10^6 cells/mL) in 24-well plates were stimulated with PMA (20 ng/mL) and ionomycin (1 µM/mL) in the presence of the protein transport inhibitor, BFA (10 µg/mL) in CM and then incubated at 37°C in a 5% CO₂ atmosphere for
4 hr. Cells were harvested and aliquots of 100 µl were stained with Mabs to CD4, CD8, and γδ T cell markers on ice followed with sheep anti-mouse IgG, F(ab’)_2-FITC conjugate (1:100) as described in the previous section. Mouse IgG1 was set up as a background staining control. After staining, cells were washed twice with chilled FACS washing buffer, fixed with 300 µl of 4% paraformaldehyde at 4°C for 20 min, blocking with 2% of normal mouse serum for 15 min, and then washed with permeabilization buffer for three times at 4°C for 3 min. The cells were pelleted and stained with 35 µl of mouse anti-porcine IFN-γ PE (1:200) Mab at 4°C for 30 min. After three washes with chilled FACS washing buffer, cells were refixed in 1% paraformaldehyde in PBS. Samples were collected by flow cytometer. Lymphocytes were gated on forward scatter (FSC) and side scatter (SSC) dot plots and ten thousand events collected. Analysis gates were set on CD4, CD8 or γδ TCR-positive cells according to the FITC emission (FL-1) and SSC. The isotype control was used to verify the staining specificity and as a guide for setting markers for negative population.

Pathological examinations: At the end of the experiment, pigs were euthanized for pathological examinations. The visceral organs were fixed in 10% neutral buffered formalin. Wet tissues were processed and embedded in paraffin wax. Sections were cut and stained with haematoxylin and eosin for the histopathological examination.

Statistical analysis: Statistical analysis of the data was calculated using analysis of variance (ANOVA) and Dunnett’s multiple-range tests with P value of < 0.05 being used to determine significance.

RESULTS

Effect of SCE administration on clinical signs and growth performance during PrV infection: Clinical signs and feed consumption were recorded daily after PrV challenge. Both SCE-treated and untreated control pigs showed a transient and mild illness between day 3 and day 12 after PrV challenge. The clinical scores included an assessment of reduced liveliness, coughing, increased nasal discharge, eyelid edema (conjunctiva reddened, clear secretion), and ruffled hair. Figure 1 shows the averages clinical scores, feed consumption and average daily gain in body weight between the untreated controls and the SCE-treated group from day 0 to 14 after PrV challenge. Both groups showed the most serious illness and reduction of feed consumption at day 6 post PrV challenge. There was a difference (p<0.05) in clinical scores between the SCE-treated (reduced score) and the control groups on the third and fourth day after PrV challenge (Fig. 1A). Although the difference in feed consumption between the SCE-treated and untreated control groups was not significant (p>0.05) (Fig. 1B), there was a slight improvement (12% increase) of average daily weight gain in pigs fed with SCE at 14 days after PrV challenge. However, this was not significantly different to the controls (p>0.05) (Fig. 1C). The feed conversion rate (FCR) in SCE-treated and control pigs from day 0 to 14 after PrV challenge were 2.47 and 2.64, respectively.

Effect of SCE administration on the cytotoxic activity of NK cells during PrV infection: The cytotoxic activity of NK cells in SCE-treated (PrV challenge with SCE administration) group showed a 91.0% and 78.5% increase in the first and second week post PrV infection, respectively, as com-
pared with the controls (PrV challenge without SCE administration) group (p<0.05) (Fig. 2).

Effect of SCE administration on phagocytic activity of monocytes and neutrophils during PrV infection: The effect of SCE on the phagocytic activity of monocytes and neutrophils was evaluated in this research. The phagocytic index of monocytes from pigs fed with SCE additive showed a 52.3% increase by the second week post PrV challenge, which was different from the controls (p<0.05). However, the enhancement was not seen in pigs fed with SCE at the first week post PrV challenge as compared with the control pigs (Fig. 3A). There was no difference in the phagocytic activity of neutrophils (p>0.05) between the control group and SCE-treated group at the first and second week post PrV challenge (Fig. 3B).

Effect of SCE administration on lymphocyte proliferation during PrV infection: The effect of SCE on T cell activation was assayed by lymphocyte proliferation to mitogen. The stimulation index from pigs fed with SCE additive showed a 76.2% increase at the first week post PrV challenge, which was different from the controls (p<0.05). However, the enhancement was not seen in pigs fed with SCE at the second week post PrV challenge as compared with the control pigs (Fig. 4).

Effect of SCE administration on interferon-gamma production during PrV infection: To address the effect of SCE on the production of IFN-γ in T cell subsets, dual staining for surface marker against CD4, CD8, and γδ T cells and intracellular IFN-γ was used to evaluate the activation of PBMCs in response to PMA and ionomycin stimulation. The percentage of IFN-γ producing cells in CD4, CD8 and γδ TCR positive cells from SCE-treated pigs showed no obvious difference compared with the control pigs (p>0.05) at the first week post PrV challenge (Fig. 5A). However there was an increase in the percentage of IFN-γ producing cells in the CD4+ and γδ TCR positive subsets from pigs fed with SCE additive at the second week post PrV challenge.

Fig. 2. Effect of SCE administration on the cytotoxic activity of natural killer cells after PrV challenge. NK cell cytotoxicity was performed in the first and second week after PrV challenge. Asterisk (*) indicates a difference (p<0.05) between the control group and the SCE-treated group.

Fig. 3. Effect of SCE administration on the phagocytic activity of monocytes (A) and neutrophils (B) after PrV challenge. Analyses of the phagocytic activity of monocytes and neutrophils were performed in the first and second week after PrV challenge. Asterisk (*) indicates a difference (p<0.05) between the control group and the SCE-treated group.

Fig. 4. Effect of SCE administration on lymphocyte proliferation after PrV challenge. Lymphocytes were stimulated with Con A and their proliferation determined. This was performed in the first and second week after PrV challenge. Asterisk (*) indicates a difference (p<0.05) in T cell activation between the control group and the SCE-treated group.
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(23.4% and 52.4% increase, respectively), both different from the controls (p<0.05). However, this enhancement of expression of IFN-γ did not occur in CD8⁺ lymphocytes (Fig. 5B).

Effect of SCE administration on pathological lesions of pigs challenged with PrV: At the end of the experiment, two pigs from each group were randomly selected and sacrificed for pathological examinations. There were no apparent gross and microscopic lesions noted in tonsil, lymph nodes and visceral organs in control and SCE-treated pigs. However, the severity of non-suppurative encephalitis characterized by heavy lymphocytic perivascular cuffing and the numbers of cuffing in cerebrum and cerebellum were obviously reduced in SCE-treated pigs (Fig. 6).

DISCUSSION

In this experiment, we demonstrated that the innate immunity of growing pigs can be up-regulated by SCE, which showed increased early resistance to PrV infection. SCE is a compound of extracts from sugar cane that contains functional ingredients including polyphenols and some unidentified ingredients that can enhance or regulate biological activities [17, 23]. In this experiment, we used anti-PrV gE antibody negative growing pigs challenged with PrV to evaluate the immunomodulatory effect of SCE. To avoid the stress of handling that might disturb leukocyte functional assays, temperature measurements to record fever were not taken during daily observation. After PrV infection, pigs in both SCE-treated and control groups all developed clinical signs including ruffled hair, decreased activity, increased nasal discharge, conjunctivitis and coughing at the third or the fourth day after PrV challenge. Feed consumption also markedly decreased between 4 and 6 days after PrV challenge. These signs were similar to those previously reported, but the severity of the clinical signs and the effect on feed consumption were not as serious as previously reported [12]. The virus virulence and dose inoculated, as well as age and immune status of pigs may contribute to the difference in this study [16]. The in vitro functional data presented here and published work suggest that SCE has the potential to up-regulate innate immunity, and it may be this which restricts PrV replication and reduces the severity of clinical disease early after PrV infection.

Natural killer cells play an important role in innate immunity for elimination of many intracellular pathogens. The activity of NK cells against pathogens is mediated by the destruction of infected cells and secretion of IFN-γ. Early IFN-γ production regulates the early adaptive immune response [6, 27], and is critical for activation of macrophages and cytotoxic T cells [18, 27]. Therefore poor NK cell function is correlated with the susceptibility to viral and other microbial infections [18]. It has been shown that the cytotoxic activity of NK cells plays an important role against PrV infection [22]. In this experiment, the NK activ-
ity of pigs fed with SCE was enhanced in the first and second week after PrV infection. A previous study has reported a strong cell mediated immune response gradually developing 3 days after PrV infection [15], in which the activated T cells specific for PrV antigen may release more cytokines to amplify NK cell cytotoxicity [3]. This may explain why the NK cell cytotoxic activity in pigs recovering from PrV infection here showed enhanced activity for two weeks in SCE-treated animals. These results suggest that administration of SCE can enhance the cytotoxic activity of NK cells that may subsequently increase the protection against early PrV infection.

Increased phagocytosis by phagocytes has been reported in chickens after SCE administration [11]. Here, the significant enhancement of phagocytic activity of monocytes was only noted in the second week in this experiment, but was not observed in the first week after PrV infection. Activation of the phagocytic activity of monocytes may be mediated by activation of virus-specific lymphocyte and so cytokine production which amplifies monocyte function in SCE-treated pigs. Here, the phagocytic activity of neutrophils was not significantly affected by SCE, which is consistent with our previous study [21], but it is different from the results observed in cultured chicken neutrophils treated with SCE after SCE treatment of chickens [11]. The reason of the discrepancy is not clear, but species differences between pigs and chickens, different approaches in SCE administration (in vivo versus in vitro), different methods into assessing phagocytic activity, and the high variation in phagocytic activity of neutrophils in pigs may contribute to the difference [7]. However, the enhancement of phagocytosis of monocytes in pigs and neutrophils in chickens [11] suggest that SCE may elicit a protective effect against bacterial infections.

Activated T lymphocytes produce a cocktail of cytokines. IFN-\( \gamma \) is one of the most important cytokines as it mediates immunoregulation and anti-viral activity of cell-mediated immunity [22]. Therefore, it is important to know whether SCE administration can enhance the activation and IFN-\( \gamma \) production of T lymphocytes. SCE treatment caused an increased response of lymphocytes to mitogen in the first week post PrV infection, but the difference was not observed in the second week. In contrast, increasing IFN-\( \gamma \) production by CD4\(^+\) and \( \gamma \delta \) T cells was only noted in the second week after PrV infection, but was not seen at all in the CD8\(^+\) subpopulation. The inconsistency in timing in the responses may be due to the sensitivity of each assay and the interference by PrV. However, these results suggest that the administration of SCE could enhance T cell activation and IFN-\( \gamma \) production, which in turn will activate macrophages and NK cells against infections. The time of the increase in T cell IFN-\( \gamma \) production fits well with the increased macrophage activity shown here. The enhancement of IFN-\( \gamma \) production may also explain the adjuvant effect of SCE, which has been reported in chickens [11]. Therefore, SCE administration enhanced NK cell activity combined with the development of T cell activation, and IFN-\( \gamma \) production may contribute to the early restriction of PrV replication in pigs and so reduced clinical signs and encephalitic lesions. The similar result of anti-viral activity, reduction in seroconversion rates in SCE treated pigs, via increasing NK activity was also noted in a previous report [21].

Studies on chickens indicate that SCE can act as adjuvant [10] and has a protective effect against *Eimeria tenella* infection [9]. *In vitro* study also reveals that SCE can significantly increase numbers and phagocytic function of polymorphonuclear cells [11]. Moreover, SCE can rescue the cell-mediated and humoral immunity in immune suppressed chickens that have been pretreated with x-irradiation [2] or cyclophosphamide [8]. These results indirectly suggest that SCE has positive effect on the activation of B and T cells, and polymorphonuclear cells, and the displays protective and adjuvant effects against infections. Similarly, the positive regulation of leukocyte functions including NK cells, lymphocytes, and monocytes, and adjuvant effect on porcine immunity were also noted in this and previous reports [21]. Therefore, SCE has a wide immunostimulating activity which can enhance innate immunity against various infections.

As the components of SCE, besides polyphenol [17, 26], were not clearly identified, the mechanisms by which SCE contributed to immunostimulatory effects requires further elucidation. However, the present study and our previous reports [21] have shown that SCE can enhance leukocyte functions and IFN-\( \gamma \) production of lymphocytes, suggesting a cytokine regulation network initiated by the components of SCE, which activates early innate immunity and adapt acquired immune response, ultimately resulting in the immunostimulatory effects.

Taken together, SCE has a positive immunoregulatory effect on porcine innate immunity, lymphocyte activation, and IFN-\( \gamma \) production that may contribute to the restriction of PrV infection. The restriction of PrV replication was consistent with decreasing the appearance of clinical features and the severity of encephalitic lesions in SCE-treated pigs. Therefore, besides the improvement of managerial and environmental systems, the administration of SCE in feed may be beneficial in reducing the effect of viral infections of PRDC in pigs and also reducing the abuse of antibiotics in disease control.

ACKNOWLEDGEMENTS. This study was supported by a grant (93AS-1.8.1-BQ-B1) from the Council of Agriculture of Taiwan, Republic of China and partial financial support was from Shin Mitsui Sugar Co., Ltd., Japan. The authors would like to thank Dr. B. A. Blacklaws for revising the manuscript and helpful discussion.

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