Effects of Active Egg White Product on Neutrophil Function in Calves

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ABSTRACT. The effects of an active egg white product (AEWP) on neutrophil function in calves were investigated. Calves were administered AEWP orally at doses of 250 mg and 500 mg/kg either once or twice, with an interval of 5 days between doses in the latter case. The peripheral blood neutrophils of calves receiving a single 500 mg/kg dose displayed increased nitroblue tetrazolium reducing activity and increased intracellular killing of *Staphylococcus aureus* from 1 day after administration, with maximum levels being attained on the 3rd day. However, no such increase of these activities was observed after administration of 250 mg/kg. Calves receiving two doses of 500 mg/kg displayed the same changes as seen in the corresponding one-dose group, while the neutrophil activity of calves receiving 250 mg/kg also increased after the second dose. However, no increase in the peripheral blood neutrophil count was observed after AEWP administration. Thus, AEWP enhanced the nonspecific antibacterial activity of neutrophils when given to calves by the oral route.—KEY WORDS : active egg white product, calf, intracellular killing activity, neutrophil, nitroblue tetrazolium reduction.

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A wide variety of antibacterial agents, mainly antibiotics, are employed for the prevention and treatment of infection in livestock. However, the administration of these drugs causes various problems such as residue and the emergence of bacterial resistance. In recent years, depressed immune function attributable to stress has caused a marked increase in the incidence of opportunistic infections, so the enhancement of host defence mechanisms against infections is currently a major field of study.

Since their specific immunity is incomplete, calves are regarded as particularly susceptible to infections such as pneumonia and diarrhea. Until cellular and humoral immunity is established, the nonspecific activity of macrophages (particularly neutrophils) plays a vital role in calf defence mechanisms[10].

In the present study, an active egg white product (AEWP), which has been found to enhance nonspecific host defence mechanisms against bacterial infection in mice [2], was administered orally to calves and changes in the peripheral blood neutrophil count were observed. Nitroblue tetrazolium (NBT) reduction and intracellular killing of *Staphylococcus aureus* (*S. aureus*) by neutrophils were also assessed to investigate the effects of AEWP administration.

MATERIALS AND METHODS

Preparation of AEWP: AEWP was prepared as described previously [2]. In brief, chicken egg white was fermented with yeast and spray dried. Lots of dried egg white powder were tested by assaying their protective effect against bacterial infection in mice, and only active lots were used as AEWP. AEWP (Neurich®) was supplied by Eisai Co., Ltd. (Tokyo, Japan).

Protection assay of AEWP: Protection assay was performed according to the method described previously [1]. Briefly, mice were administered orally with lots of dried egg white powder emulsified with distilled water 1 day before *Escherichia coli* infection. As a control, mice were orally given the same volume of distilled water instead of these products. Mice were inoculated intravenously with 5 × 10^7 CFU of *E coli* E0192, a clinical isolate from humans. The protective effect of these products was determined by measuring the survival rate after 7 days, and only active lots were selected as AEWP.

Calves: Eighteen healthy male Holstein calves aged 2–3 weeks were divided into six groups of three calves each. All the calves had been raised at a farm on the outskirts of Obihiro (Hokkaido, Japan). AEWP was administered either as a single dose or as two doses at an interval of 5 days. In all cases, it was administered orally mixed with a milk replacer. The doses of AEWP used were 250 mg/kg, 500 mg/kg, or zero (control group) in both the single-dose and two-dose groups.

Leukocyte and neutrophil counts: Peripheral blood leukocytes were counted with a haemocytometer. Neutrophil counts were estimated from the differential count determined using Giemsa-stained blood smears.

Isolation of neutrophils: Neutrophils were isolated by a modification of the method of Roth and Kaeberle [12, 14]. Specifically, 15 m*l* of heparinized blood was layered onto a Ficoll-Paque solution (Pharmacia Fine Chemicals, Sweden) and centrifuged at 20°C and 425 × g for 30 min. Then the plasma, monocyte, and Ficoll-Paque layers were removed by aspiration, and 30 m*l* of 0.83% ammonium chloride was added to the remaining erythrocyte-neutrophil layer to lyse the erythrocytes. This specimen was then centrifuged at 200 × g for 10 min, and after removing the supernatant by aspiration, the residual neutrophil sediment was washed twice with cold modified Hank's balanced salt solution (HBSS; Sigma Chemical Co., U.S.A.). The recovered neutrophils were then resuspended in cold HBSS, and stained with 0.5% trypan blue to determination their viability. The final neutrophil

concentration was adjusted to 5×10^6 cells/ml.

NBT reduction assay: The NBT reduction assay was performed by a modification of the method of Roth and Kaeberle [12]. A solution was prepared by dissolving NBT (Grade III, Sigma Chemical Co., U.S.A.) in HBSS at 1 mg/ml, while a zymosan solution was prepared by dissolving Zymosan A (Sigma Chemical Co., U.S.A.) in RPMI 1640 medium (Nissui, Japan) at 10 mg/ml. To the test tubes designated for testing phagocytosis, 0.1 ml of zymosan solution, 0.4 ml of NBT solution, and 0.5 ml of cell suspension were added. To the test tubes labelled "resting", 0.1 ml of RPMI 1640 was added instead of the zymosan solution. All tubes were shaken for 30 min in a constant temperature bath at 37°C, after which 1 ml of 0.5 N hydrochloric acid was added to terminate the reaction. Then, after centrifugation at 1,000 × g for 10 min, the supernatant was discarded, and the cells were resuspended in 3 ml of dimethyl sulphoxide (DMSO). The cell suspension was next transferred to a glass test tube and heated in a boiling water bath for 5 min to elicit formazan coloration. Then the specimen was let stand at room temperature, after which the optical density (OD) of the mixture was measured at 565 nm with DMSO as the blank. The measured values were corrected by subtracting the OD for HBSS plus the NBT reagent in the case of resting specimens, while the value of the zymosan solution was also subtracted in the case of phagocytic specimens. The phagocytic value minus the resting value was used as an index of phagocytic activity.

Intracellular killing assay: Tests of intracellular killing activity were conducted in accordance with a modification of the method of Hori [5]. Briefly, 3.6 ml of HBSS, 0.4 ml of normal bovine serum, and 0.5 ml of a suspension of *S. aureus* 209P (2.5×10^7 cells/ml) were mixed with 0.5 ml of the neutrophil suspension. Prior to incubation (0 min) as well as after 90 min of shaking culture at 37°C, 0.5 ml samples were taken and serially diluted $10–10^5$ times with physiological saline. A 0.1-ml aliquot of each diluted sample was mixed with 15 ml of nutrient agar medium in a Petri dish and incubated at 37°C for 48 hr, after which the number of *S. aureus* 209P colonies was counted. The ratio of the surviving bacteria in the sample incubated for 90 min to that in the 0-min sample was determined as an index of intracellular killing activity.

Statistical analysis: Student's t-test was used for the statistical analysis of the data.

RESULTS

Neutrophil count and neutrophil function following a single dose of AEWP: The peripheral blood neutrophil count in the calves receiving a single dose of AEWP (250 mg/kg or 500 mg/kg) displayed no marked changes after administration, and showed no significant difference from the corresponding values for the control group (Fig. 1).

The changes in NBT reducing activity are shown in Fig. 2. In the group receiving a single 500 mg/kg dose of AEWP, elevation of activity was observed from the 1st
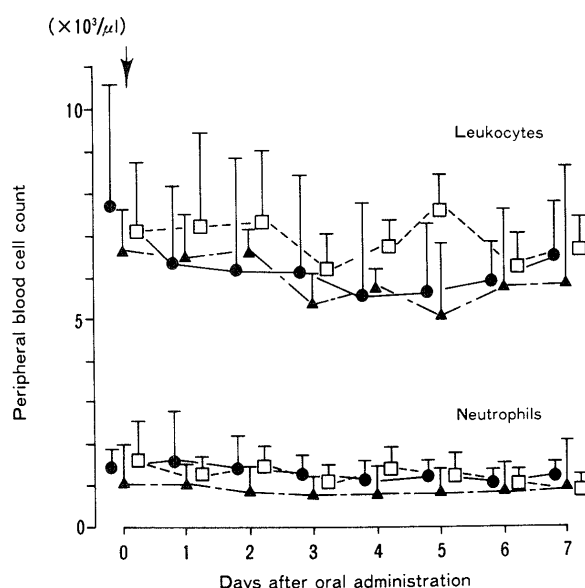

Fig. 1. Effect of a single dose of AEWP on the peripheral blood leukocyte and neutrophil counts. ↓ oral administration of AEWP, ● — ● 500 mg/kg of AEWP, ▲---▲ 250 mg/kg of AEWP, □---□ control.
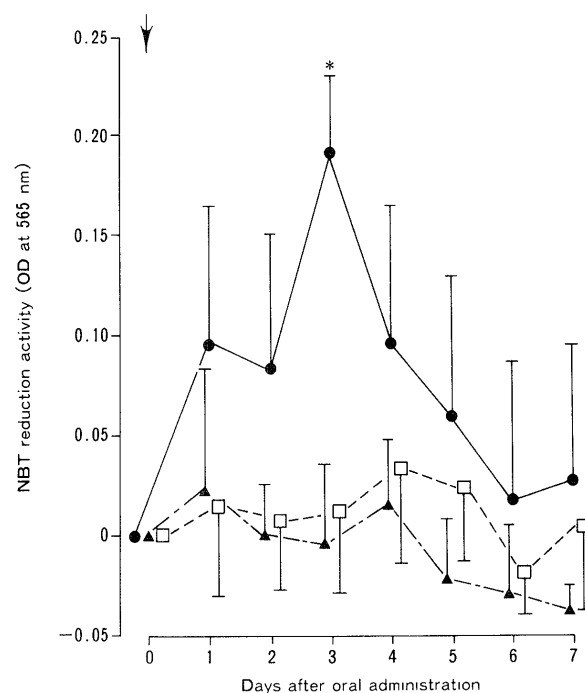

Fig. 2. Effect of a single dose of AEWP on NBT reduction activity. ↓ oral administration of AEWP, ● — ● 500 mg/kg of AEWP, ▲---▲ 250 mg/kg of AEWP, □---□ control. Significant difference from control: *, P<0.05.

day after administration. NBT reducing activity reached its maximum value (P<0.05) on the 3rd day, subsequently diminished, and returned almost to the preadministration level on the 6th day. In contrast, the group receiving a single 250 mg/kg dose showed no significant changes in

NBT reduction after administration, and the activity levels were similar to those of the control group.

Changes in the intracellular killing of *S. aureus* are shown in Fig. 3. In the 500 mg/kg group, the bacterial survival ratio was decreased significantly (P<0.05) on the 1st day after administration when compared to before administration (44.2 ± 2.5%), and it reached a minimum (18.6 ± 2.0%) on the 3rd day, indicating a marked enhancement of intracellular killing activity. The killing activity returned towards preadministration levels on the 5th day. In contrast, no significant changes were noted in the calves receiving a 250 mg/kg dose.

Neutrophil count and neutrophil function after two doses of AEWP: As in the single-dose experiments, the peripheral blood leukocyte and neutrophil counts in the calves receiving two doses of AEWP showed no changes after administration (Fig. 4).

The changes in NBT reducing activity following 2 doses of AEWP are shown in Fig. 5. In the 2 × 500 mg/kg group, enhancement of NBT reducing activity was noted on the 1st day after administration. Activity reached a peak (p<0.05) on the 3rd day and diminished thereafter, with the same trend being observed after administration of the 2nd dose. In contrast, the 2 × 250 mg/kg group showed no elevation of activity after the 1st dose. However, following the 2nd dose a significant elevation (p<0.05) was noted on the 2nd day and a high level was also maintained on the 3rd day.

Changes in the intracellular killing of *S. aureus* are shown in Fig. 6. In the calves receiving 2 × 500 mg/kg

Fig. 4. Effect of two doses of AEWP on the peripheral blood leukocyte and neutrophil counts. ↓ oral administration of AEWP, ● — ● 500 mg/kg of AEWP, ▲---▲ 250 mg/kg of AEWP, □---□ control.

Fig. 5. Effect of two doses of AEWP on NBT reduction activity. ↓ oral administration of AEWP, ● — ● 500 mg/kg of AEWP, ▲---▲ 250 mg/kg of AEWP, □---□ control. Significant difference from control: *, P<0.05; **P<0.01.
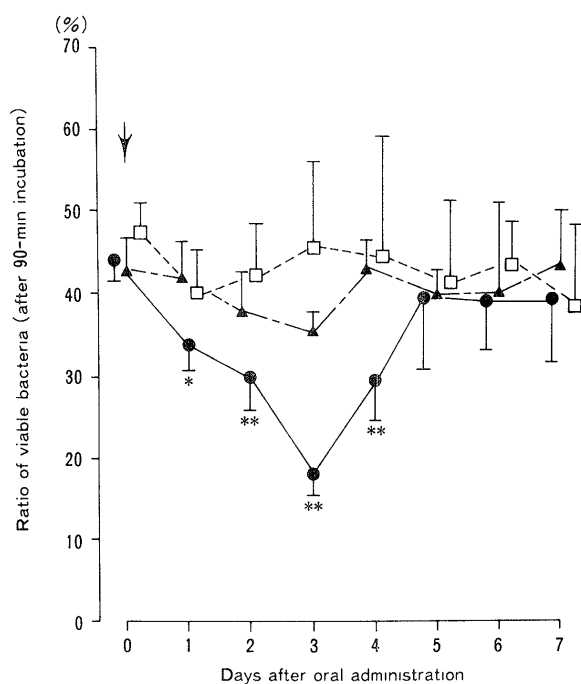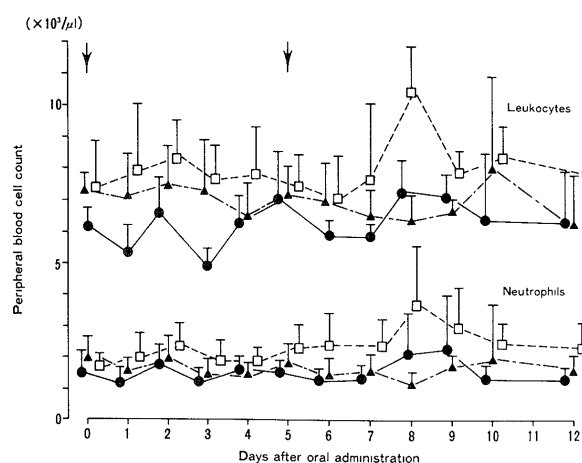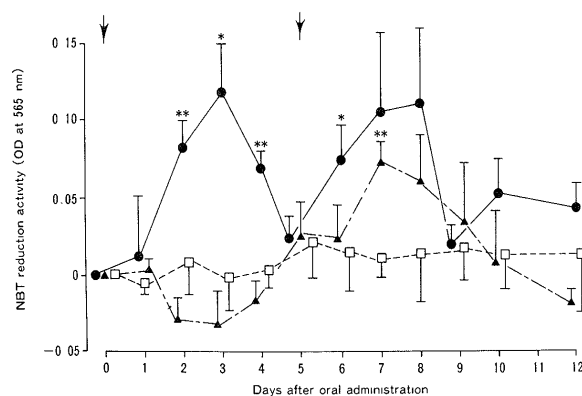
Fig. 3. Effect of a single dose of AEWP on the bactericidal activity of neutrophils against *Staphylococcus aureus* strain 209P. ↓ oral administration of AEWP, ● — ● 500 mg/kg of AEWP, ▲---▲ 250 mg/kg of AEWP, □---□ control. Significant difference from control: *, P<0.05; **P<0.01.
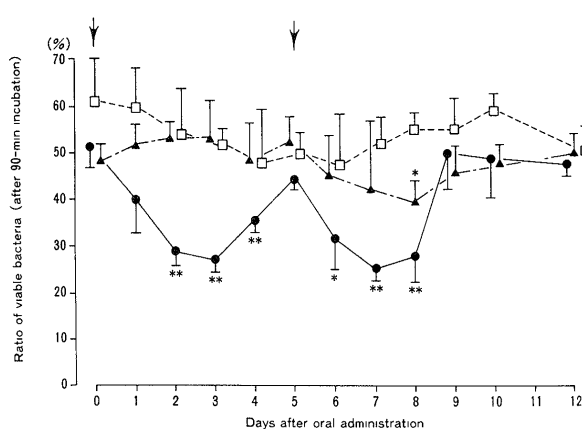
Fig. 6. Effect of two doses of AEWP on the bactericidal activity of neutrophils against *Staphylococcus aureus* strain 209P. ↓ oral administration of AEWP, ● — ● 500 mg/kg of AEWP, ▲---▲ 250 mg/kg of AEWP, □---□ control. Significant difference from control: *, P<0.05; **P<0.01.

dose, the bacterial survival ratio was 51.0 ± 4.6% before administration and decreased to a minimum of 26.5 ± 2.7% (p<0.01) at 3 days after administration, indicating a marked enhancement of intracellular killing activity. Moreover, after the 2nd dose the killing activity displayed changes similar to those observed subsequent to the 1st dose, and in fact exhibited changes similar to those in the calves receiving a single dose. On the other hand, in the 2 × 250 mg/kg group, no change was recognized after the 1st dose. After the 2nd dose, killing activity rose and reached a maximum (p<0.05) on the 3rd day, displaying the same trend as the changes in NBT reducing activity.

DISCUSSION

The AEWP used for the present study was a crude natural product prepared from hen's eggs. Oral administration of this product to calves elicited a clear enhancement of peripheral blood neutrophil function at 1–4 days after administration. Previously known activators of neutrophil function include levamisole, lysostaphin, tuftsin, azimexon, and muramyl dipeptide [7, 9, 11, 16]. All of these substances are believed to promote the phagocytic activity of neutrophils by increasing the intracellular cGMP concentration. The neutrophil-activating substance in AEWP has not been identified. However, since the activation of peripheral blood neutrophils was achieved by oral administration, the stimulatory effect was possibly due to some factor contained in this product itself or a peptide produced by the hydrolysis in the digestive tract, or else to the indirect action of substances taken up and metabolized from the AEWP by neutrophils.

Superoxide anions (O_2^-) are produced following the binding of opsonized foreign particles to Fc or C3b receptors on neutrophils, or even with unopsonized particles, by the stimulation of fMet-Leu-Phe (fMLP) receptors. Then further conversion of O_2^- to other forms of active oxygen such as hydrogen peroxide or hydroxyl radicals occurs, indicating the activation of the potent bactericidal mechanism constituted by the hydrogen peroxide-myeloperoxidase-chloride system [6]. The NBT reducing activity test takes advantage of the fact that NBT is reduced to formazan by neutrophil O_2^- [3]. Since clear enhancement of NBT reducing activity occurred after the administration of AEWP in the present study, this substance is presumed to have activated the oxygen-dependent bactericidal system of neutrophils. Moreover, the increased intracellular killing of *S. aureus* that occurred along with the changes in NBT reducing activity also indicated that oral AEWP was effective in activating neutrophils in calves.

Administration of a single 500 mg/kg dose of AEWP was effective in activating neutrophils, but no elevation of activity was observed after a single 250 mg/kg dose, indicating that this substance activated neutrophils in a dose-dependent manner. When two doses were administered at an interval of 5 days, the 250 mg/kg dose level also caused significant neutrophil activation after the 2nd dose,

suggesting that the efficacy of AEWP could be enhanced by continued administration at low doses. Determination of the most effective administration schedule for this product requires further study in the future.

Drug such as antibiotics or synthetic glucocorticoids are commonly employed for the treatment of infections in calves. However, administration of antibiotics is believed to depress rather than improve neutrophil function [8], while administration of glucocorticoid has an immunosuppressive effect [15] and has also been shown to depress intracellular killing of *S. aureus* or NBT reducing activity [13]. Furthermore, stress increases the blood concentration of endogenous glucocorticoids, thereby inducing immunosuppression, and is reported to depress antibody titers [4]. Therefore, the role of drug therapy in the treatment of infections in calves is being reconsidered, with the enhancement of immunological function coming to be regarded as an essential factor. Use of AEWP in the treatment of calves to prevent infection-related losses appears highly promising, and deserves further investigation.

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