Aloe-emodin has shown anti-neoplastic activity against some human cancer cell lines. This study aimed to explore the effects of aloe-emodin on the phagocytosis of macrophages, the activity of natural killer (NK) cells and the expression of cytokines in leukocytes from Sprague-Dawley rats. Leukocytes were collected, placed into culture plates and the functions of macrophages and NK cells and the percentage of viable cells were determined by flow cytometric analysis. Incubation of leukocytes with various concentrations of aloe-emodin caused a dose-dependent decrease of viable cells, a decrease of phagocytosis by macrophages, and a decrease of the activity of NK cells. Evaluation of cytokines in leukocytes by ELISA indicated that aloe-emodin increased the levels of interleukin (IL)-1β and tumor necrosis factor (TNF)-α. The results were also confirmed by PCR assay for the mRNA expression of the examined cytokines.

There is much evidence that inflammatory responses are associated with cytokines which are released from leukocytes. Cytokines are soluble hormone-like protein mediators, produced by diverse cell types in response to various stimuli including other cytokines. The most important cytokine activities are associated with the immune response, inflammation, tissue injury or repair, and organ dysfunction (1-4).

Aloe-emodin (1,8-dihydroxy-3-(hydroxymethyl)-anthraquinone) (AE) is an active component from the roots and rhizomes of Rheum palmatum and is also naturally present in the leaves of Aloe vera (5, 6). It significantly inhibited the growth of Merkel cell carcinoma cells (7), and human neuroectodermal tumor cell growth in vitro and in animal models with no evidence of acute or chronic toxicity (8). AE appears to have some protective effect not only against hepatocyte death, but also on the inflammatory response subsequent to lipid peroxidation (9). It was reported that the anti-glioma action of AE involves ERK-independent induction of both apoptosis and autophagy, as well as ERK inhibition-mediated differentiation of glioma cells (10). AE can down-regulate the anticancer activity of cisplatin by blocking the activation of ERK in tumor cells (11) and promote NK cell activity (12).

To date, no information is available on the effects of AE on the viability, the functions and the levels of cytokines of rat leukocytes, prompting this in vitro study.

Materials and Methods

Chemicals and reagents. Aloe-emodin (AE), Con A, heparin, Tris-HCl, propidium iodide (PI), ribonuclease-A, LPS, triton X-100 and trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Human IL-1, IL-1β, IL-6 and TNF-α were purchased from Calbiochem-Novachem Corporation (Germany). Dimethyl sulfoxide (DMSO), potassium phosphates and TE buffer were purchased from Merck Co. (Darmstadt, Germany). RPMI 1640 medium, fetal bovine serum (FBS), glutamine, penicillin-streptomycin and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA).

Animals. Forty-two male Sprague-Dawley (SD) rats, weighing 180-200 g were obtained from the Animal Center of NSC (Taipei, Taiwan, ROC). The animals were housed in cages and maintained at 25°C on a 12-h light/dark cycle in the Animal Center of China Medical University.
Medical University (Taichung, Taiwan, ROC), following the animal guideline and had free access to water and chow. At the time of sacrifice, all the animals were at least 12 weeks of age.

**Grouping rats for experiments.** The 42 rats were divided into 6 groups as described previously (12): Group I comprised 6 rats for cytotoxicity experiments; Group II, 6 rats for cytokine experiments; Group III, 6 rats for NK cell activity experiments; Group IV, 6 rats for phagocytosis experiments; Group V, 6 rats for polymerase chain reaction (PCR) experiments; and Group VI comprised 12 rats for control experiments.

**Isolation of leukocytes from SD rats.** Whole blood from the SD rats’ hearts was collected under anesthesia. Leukocytes were separated from the whole blood by the Ficoll-Paque procedure, as described previously (12). The leukocytes were placed into a culture flask with RPMI 1640 medium and glutamine, and were incubated at 37°C in 95% air and 5% CO2 for 10 min, followed by cell counting by trypan blue exclusion and flow cytometry for the total viable cell numbers (12).

**Treatment of leukocytes by AE for cytotoxicity determinations.** A total of 1x10^5 cells in 1 ml of RPMI 1640 medium with various concentrations of AE in dimethyl sulfoxide (DMSO) were cultured in each well of a 24-well culture plate, followed by centrifugation of the media via centrifugation. The same volume of vehicle (DMSO) was added to the controls as to the AE-treated samples. The cells were treated with AE for 12 and 24 h, before centrifugation at 1000xg for 5 min to remove the medium. The cells were counted by trypan blue exclusion and flow cytometry for the determination of percentage of viable cells (12, 13).

**Treatment of leukocytes by AE for examining the levels of cytokines.** About 1x10^5 cells in 1 ml of medium with AE were cultured for 1, 2 or 6 h in each well of a 24-well culture plate, and Con A or LPS were also cultures as positive control, followed by centrifugation at 1500g for 5 min to remove the cells and debris, so that the supernatants could be subjected to assays. The levels of interferon (IFN)-γ, tumor necrosis factor TNF-α, IL-1β, IL-2 and IL-6 were quantified using the following kits (all from R & D Systems, USA): Quantikine human IFN-γ Immunoasay kit, Quantikine human TNF-α Immunoasay kit, Quantikine human IL-1β Immunoasay kit and Quantikine human IL-6 Immunoasay kit, respectively. Assays were performed according to manufacturer’s recommended procedures (12).

**Reverse transcription polymerase chain reaction (RT-PCR) for determining cytokine gene expression.** The total RNA was extracted from leukocytes which had been treated with or without 4, 40 and 200 μM AE for 24 hours by using Qiagen RNeasy Mini Kit, as described previously (12). A 1.5 μg RNA, 0.5 μg of oligo-dT primer and DEPC (diethyl pyrocarbonate)-treated water were combined into a 0.5 μL microcentrifuge tube (final volume, 12.5 μL). The entire mixture was heated at 70°C for 10 min and chilled on ice for at least 1 min. The subsequent procedures for conducting reverse transcription were exactly the same as those in the instruction manual (First-strand cDNA synthesis kit, Novagen). The sequences of primers are as follows: TNF-α: 3’ primer 5’-CTAAGTCTTTGACTTGTAC-3’ and 5’ primer 5’-GGTAATC CATCCTGTCAGAA-3’; IL-6: 3’ primer 5’-CAGAGCATGGGA AGT TGG GG-3’ and 5’ primer 5’-CTTCCAGCCATGGCCTTTCTTT-3’ (496 bp); INF-γ: 3’ primer 5’-TTACAGATCTTTGTGAGCCA CCG-3’ and 5’ primer 5’-GACAAATCGGCAA GCCTTGT-3’ (192 bp). Under optimized PCR conditions, all data were collected without saturation or missing bands. Each assay was conducted at least twice to ensure reproducibility (14-16).

**Statistics.** The data were expressed as mean±S.D. Differences between the control and AE-treated groups were analyzed using the Student’s t-test and the two-way analysis of variance (ANOVA) with replication. A p-value<0.05 was considered to be significant.

**Results**

**Effects of various concentrations of AE on leukocyte viability.** The results indicated that the number of viable leukocytes
decreased as the time and concentration of AE increased (Figure 1A and B).

**Effects of various concentrations of AE on the activity of NK cells.** The results indicated that the target cells were killed by NK cells, but when the concentration of AE increased from 40 to 400 μM, it led to increased NK cell activity, suggesting that AE affected the NK cell activity in a dose-dependent manner (Figure 2).

**Effects of various concentrations of AE on macrophage phagocytosis.** The results indicated that the target cells were phagocytized by macrophages at 1 h treatment with AE in a dose-dependent manner, but increased AE treatment time led to a decrease in phagocytized target cells (Figure 3).

**Effects of various concentrations of AE on cytokine release from leukocytes.** Only low levels of IL-1β and TNF-α were detected in the media of the control. However, IL-1β and TNF-α were secreted from 40 μM AE-treated cells. The secretions of IL-1β and TNF-α were dose-dependent (Figure 4A, B, C and D), but there were no significant differences for IL-2 between the untreated and treated groups (data not shown). IL-6 and INF-γ were not affected by AE. However, the Maco -1 marker increased with increasing concentrations of AE, indicating that AE promoted the differentiation of macrophages (data not shown).

**Effects of AE on gene mRNA expression in rats leukocytes.** The mRNA gel picture of cytokines with or without AE treatment and ratio of the cytokine mRNA levels in response to 40 and 100 μM AE on leukocytes is presented in Figure 5A and B. Figure 5 shows that IL-1β and TNF-α mRNA levels increased after AE was added to the examined cells for 24 h, but did not affect IL-2 and inhibited the expressions of IL-6 and INF-γ (data not shown).

**Discussion**

AE is one of the major components of *Rhei Rhizoma*, used for the treatment of various liver diseases in traditional Chinese medicine. It has been reported that AE can selectively inhibit human neuroectodermal tumor cell growth and lacked acute or chronic toxicity in an animal model (8). Here, the effect of AE on leukocytes was examined and cytokine gene expression after AE treatment was studied. The results provided further support that AE may act as an anti-inflammatory drug. The data presented here established that AE decreased the percentage of viable leukocytes, promoted NK cell activity and macrophage phagocytosis, increased the release of IL-1β and TNF-α, but promoted macrophage differentiation, as judged by the increased percentage of cells labelled by anti-Maco-1. This is in agreement with other reports that demonstrated that AE inhibited the growth of human cancer cell lines in vitro (8, 9, 11). Additionally, we found that AE induced apoptosis in cancer cell lines based on the appearance of the sub-G1 group in cell cycle analysis and DAPI staining (data not shown), also agreeing with the above (8, 9, 11).

Other laboratories have demonstrated the chemopreventive and cytotoxic in vitro effects of AE in many cell types (17-19) and in animal models, with no evidence of acute or chronic toxicity to the animals (7). It was also shown that, the mechanism behind AE’s mode of action involves a
receptor-mediated recognition process for AE uptake in neuroectodermal tumor cells (7). Recently, it was reported that AE has capacity to directly kill tumor cells, but also to protect them from NO-mediated toxicity (10).

In the macrophage activity experiments, AE increased the cell phagocytizing activity. The percentage of target cells being phagocytised increased with increasing concentration of AE in the culture media for 1 h, however, increased...
treatment time led to a decrease in phagocytosis. Both effects were dose- and time-dependent.

In the cytokine studies, induction of IL-1β and TNF-α secretion in AE-treated leukocytes-derived from SD rats’ blood was detected, while IL-1β, TNF-α and IFN-γ mRNA were detected and increased in AE-treated leukocytes. To our knowledge, the induction of IL-1β and TNF-α secretion in AE-treated leukocytes has not been reported previously. Our results suggest that leukocytes may be the target cells for cytokines but they also produce cytokines and stimulate other types of cells, if promoted by AE. Other investigators had reported that TNF-α acts as a primer to sensitize hepatocytes to the proliferative effects of growth factors and offers a mechanism to explain the initiation and progression phases of liver regeneration after partial hepatectomy (20). AE had been showed to have some protective effect not only against hepatocyte death, but also on the inflammatory response subsequent to lipid peroxidation (9). Thus, we suggest that AE promoted immune cell function and the levels of cytokines in the examined cells.

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References