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Pycnogenol® Augments Macrophage Phagocytosis and Cytokine Secretion

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Abstract: We previously reported that Pycnogenol®, procyanidins extracted from the bark of French maritime pine (*Pinus maritima* Aiton; the official botanical name is now *Pinus pinaster* Aiton) is a potent free radical scavenger. It has been shown to inhibit macrophage oxidative burst. Macrophages carry out their microbicidal and tumoricidal activities via oxygen-dependent and oxygen-independent mechanisms. The present study investigated the effects of Pycnogenol® on oxygen-independent killing mechanisms of macrophages, with particular interest in phagocytosis and cytokine release. J774 cells, a murine macrophage cell line, were preincubated with Pycnogenol® and then exposed to fluorescein-conjugated *Escherichia coli* particles for phagocytosis. Pycnogenol® significantly enhanced the phagocytosis by J774 cells. Incubation with Pycnogenol® resulted in a significant increase in cell size indicating macrophage activation. J774 cells were treated with Pycnogenol® for 22 hr and the supernatants were tested for the release of tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β). Pycnogenol® significantly increased the secretion of both TNF-α and IL-1β. These results suggest that Pycnogenol® can enhance the macrophage function by increasing its ability to phagocytosis and secretion of TNF-α and IL-1β. These two cytokines may provide costimulatory signals to enhance both the humoral and cellular immune responses to promote host defense.

Key Words: Pycnogenol®, J774 murine macrophage, phagocytosis, tumor necrosis factor-α, interleukin-1β

Introduction

Macrophages play an important role in host defense by phagocytizing foreign invaders, undergoing oxidative burst, presenting antigen, and secreting cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β). Macrophages are activated in inflammatory sites (Adams and Hamilton, 1984). They can be activated by bacterial lipopolysacchride, interferon-γ, IL-12, IL-18, ultraviolet irradiation and ozone (Adams and Hamilton, 1984; Vairo *et al.*, 1992; Munder *et al.*, 1998; Virgil *et al.*, 1998). An increase in cell size, protein content, and enzyme activity has been used as markers of macrophage activation (Adams and Hamilton, 1984; Enane *et al.*, 1993). The activated or “angry” macrophages as they are sometimes called, can efficiently destroy microbes, parasites, and tumor cells. Activated macrophages have been of interest in clinical studies in local or systemic adoptive cancer immunotherapy as they can act selectively against neoplastic cells (Bartholeyns, 1993). TNF-α, known for its cytotoxic effect on tumor cells, is secreted from activated macrophages and monocytes (Currie and Bashan, 1975). IL-1β, also termed lymphocyte activating factor, has been known to protect against lethal changes brought about by radiation, hyperoxia and infection (Gupta, 1988). Through TNF-α and IL-1β macrophages control the proliferation, differentiation, and effector functions of lymphocytes (Parslow and Bainton, 1997). TNF-α and IL-1β have overlapping biological activities that produce a broad range of effects on nonhematopoietic as well as hematopoietic cells (Gupta, 1988; Oppenheim and Ruscetti, 1997). The activation of macrophages is regulated by a network of mediators. For example, TNF-α and IL-1β enhance their own release as well as that of each other (Durum and Oppenheim, 1989). These two cytokines provide costimulatory signals to enhance the activation of helper T lymphocytes and thus promote both the humoral and cellular immune responses (Oppenheim and Ruscetti, 1997).

Pycnogenol® (PYC) is a blend of water-soluble oligomeric and monomeric procyanidins extracted from the bark of French maritime pine (*Pinus maritima* Aiton; the official botanical name is now *Pinus pinaster* Aiton) (Masquelier, 1987). It has been known to reduce inflammation, and alleviate a variety of conditions linked to the deleterious action of free radicals (Passwater, 1992; Passwater and Kandaswami, 1994).

In our laboratory, we have demonstrated that PYC can protect vascular endothelial cells from injury induced by an organic oxidant, *t*-butyl hydroperoxide (Rong *et al.*, 1995). It can increase the levels of intracellular glutathione and enhance the activities of antioxidant enzymes (Wei *et al.*, 1997). PYC also inhibits macrophage oxidative burst, lipoprotein oxidation, and hydroxyl radical-induced DNA damage (Nelson *et al.*, 1998). We have shown that PYC enhances immune and hemopoietic functions and counters learning

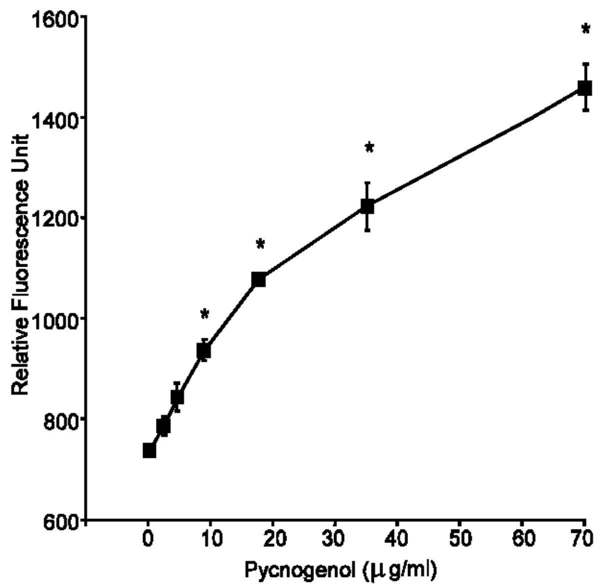

Fig.1: Effect of PYC on phagocytosis. J774 cells (10^5 cells/well) in 96-well plates were preincubated with PYC (2.18, 443, 8.75, 17.5, 35, and 70 μg/ml) in DMEM at 37 °C and 5% CO_2 for 2 hr. Following removal of PYC, fluorescein-conjugated *E. coli* particles were added and further incubated at 37 °C for 1 hr. Phagocytosis was measured with the fluorometric assay. Data represent means ± SE of triplicate samples. *Significant difference (P < 0.05) compared with control without PYC.

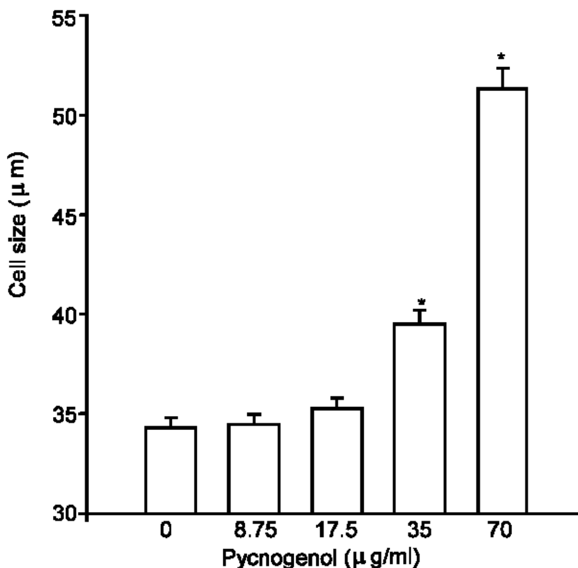

Fig. 2: Effect of PYC on cell size. J774 cells (7.4 x 10^5 cells/well) in 6-well plates were incubated with PYC (8.75, 17.5, 35, and 70 μg/ml) in DMEM at 37 °C and 5% CO_2 for 22 hr. Data represent means ± SE of three experiments. *Significant difference compared with control without PYC (P < 0.05).

impairment and memory deficit in senescence accelerated mice (Liu *et al.*, 1998; Liu *et al.*, 1999). More recently, we showed that PYC protects neurons from amyloid-β peptide-induced apoptosis (Peng *et al.* 2002). The clinical benefits of PYC have been summarized in a recent review article (Rohdewald, 2002). In the present paper, we report the effects of PYC on activation of macrophages pertaining to cell size, phagocytosis, and secretion of TNF-α and IL-1β.

Materials and Methods

Chemicals and Reagents: PYC (Pycnogenol® — a registered trade mark of Horphag Research Ltd., Geneva, Switzerland) was provided by Henkel Corporation (La Grange, IL, U.S.A.). Dulbecco's modified Eagles Medium (DMEM) and penicillin-streptomycin solution were purchased from Mediatech Co. (Herndon, VA, U.S.A.). Bovine calf serum (BCS) was from Hyclone Laboratories (Logan, UT, U.S.A.). Phosphate buffered saline (PBS) and citric acid were from Sigma Chemical (St. Louis, MO, U.S.A.). Fluorescein-conjugated *Escherichia coli* K-12 bio-particles were purchased from Molecular Probes (Eugene, OR, U.S.A.). Trypan blue was purchased from Matheson Coleman and Bell (Norwood, OH, U.S.A.). The Quantikine M mouse TNF-α Immunoassay kit was from R & D Systems (Minneapolis, MN, U.S.A.). PYC was dissolved in PBS. *E. coli* particles were suspended in PBS and sonicated before use. The bacterial number was determined visually by counting in a hemacytometer and adjusted to 10^8/ml.

Cell Line: The murine J774 macrophage cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.). Cells were grown in DMEM, supplemented with 10% BCS, 200 U/ml penicillin and 200 μg/ml streptomycin, for 3-5 days, at 37 °C in a humidified 5% CO_2 atmosphere. The viability of cells used in experiments was always greater than 95% as determined by trypan blue exclusion.

Phagocytosis Assay: The microfluorometric phagocytosis assay was performed in 96-well plates as previously described (Wan *et al.*, 1993). Suspension of fluorescein-conjugated *E.coli* particles (100 μl/well) was used to measure the ability of macrophages to phagocytize these particles with or without PYC pretreatment. The results were expressed as the relative fluorescence unit.

Cell Size Measurement: J774 cells (7.4 x 10^5 cells/well) were seeded in 6-well plates and incubated with PYC (8.75, 17.5, 35, and 70 μg/ml) in DMEM at 37 °C for 22 hr. Random fields at 320 x magnification (Zeiss axiovert 100TV, Carl Zeiss Inc., Thornwood, NY, U.S.A.) were digitized (Hamamatsu C2400 CCD, Hamamatsu

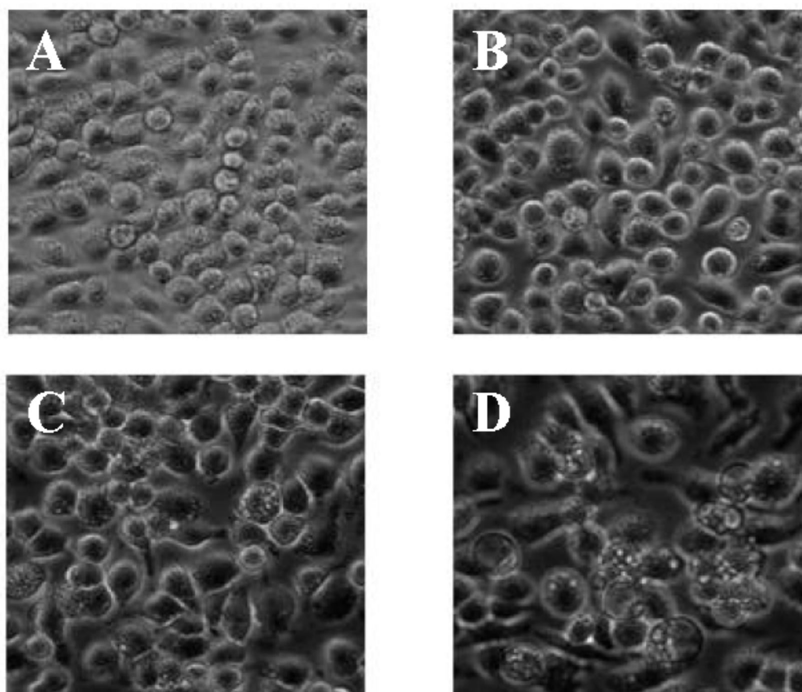

Fig. 3: Photomicrograph showing effect of PYC on cell size. Magnification 320 X. A, B, C, and D represent cells incubated with 0, 8.75, 35, and 70 µg/ml of PYC, respectively.

Corporation, Bridgewater, NJ, U.S.A.) for each experiment. Diameters of 60 cells at each PYC concentration were measured using the MetaMorph 2.5 software (Universal Imaging Corp., West Chester, PA, U.S.A.).

TNF-α Immunoassay: J774 cells (10^5 cells/well) in 96-well plates were incubated with PYC (8.75, 17.5, 35, and 70 µg/ml) in DMEM at 3 °C for 22 hr. The supernatant was collected from each well and the TNF-α levels were measured by using the Quantikine M mouse TNF-α Immunoassay kit following the manufacturer's instructions. The intensity of color of the final product is proportional to the amount of TNF-α. The absorbance was determined at 450 nm with 400 AT EIA (Whittaker Bioproducts, Walkersville, MD, U.S.A.).

Interleukin-1β: J774 cells (10^5 cells/well) in 96-well plates were incubated with PYC (8.75, 17.5, 35, and 70 µg/ml) in DMEM at 37 °C for 22 hr. The supernatant was collected from each well and stored at -20 °C until shipment. Determination of IL-1β level was performed by UMAB Cytokine Core Laboratory (Baltimore, MD, U.S.A.) using the biotin-strepavidin-peroxidase ELISA.

Statistical Analyses: Experimental data were analyzed with one-way analysis of variance (ANOVA) followed by Tukey's multiple range test for significant difference, and the results were expressed as means ± SE. Statistical significance was defined as $P < 0.05$. All statistical procedures were performed with Statgraphics software version 5.0 (STSC, Rockville, MD, U.S.A.).

Results

Each experiment was repeated at least three times with consistent results indicating that the assays used in this study were highly reproducible. Fig. 1 shows the effect of PYC on phagocytosis. PYC caused a concentration-dependent increase in the ability of J774 cells to phagocytize the fluorescein-conjugated *E. coli* particles as reflected by an increase of relative fluorescence units. Significant increases were noted between 8.75 and 70 µg/ml of PYC.

A concentration-dependent increase in cell size was observed when J774 cells were treated with PYC (Fig. 2 and 3). Statistically significant increases of 15% and 50% were noted with 35 µg/ml and 70 µg/ml of PYC, respectively (Fig. 2).

J774 cells were treated with PYC for 22 hr and the supernatants were tested for TNF-α release. A concentration-dependent increase of TNF-α release was noted with significant increases of 47 and 67% noted at 35 and 70 µg/ml of PYC, respectively (Fig. 4).

Fig. 5 shows the IL-1β release when J774 cells were treated with PYC. The IL-1β was below the level of detection up to 17.5 µg/ml of PYC. However, it increased significantly at PYC dosages of 35 and 70 µg/ml.

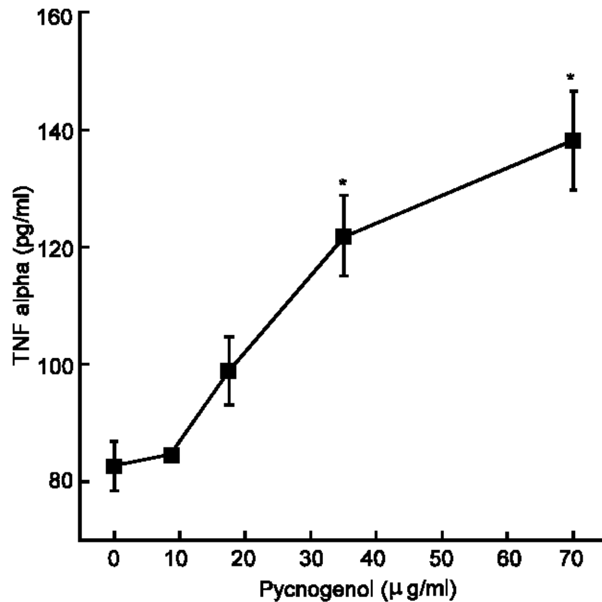

Fig. 4: TNF-α release by J774 cells. J774 cells (10^5 cells/well) in 96-well plates were incubated with PYC (8.75, 17.5, 35, and 70 µg/ml) in DMEM at 37 °C and 5% CO_2 for 22 hr. The supernatant was collected and the TNF-α release was measured. Data represent means ± SE of triplicate samples. *Significant difference compared with control without PYC (P < 0.05).

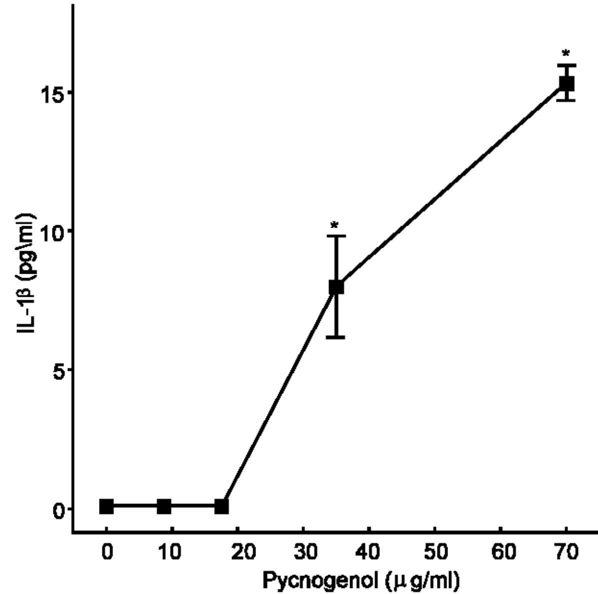

Fig. 5: Interleukin-1β release by J774 cells. J774 cells (10^5 cells/well) in 96-well plates were incubated with PYC (8.75, 17.5, 35, and 70 µg/ml) in DMEM at 37 °C and 5% CO_2 for 22 hr. IL-1β in the supernatant was measured. Data represent means ± SE of triplicate samples. *Significant difference from control without PYC (P < 0.05).

Discussion

Macrophages constitute a major part of the host defense system against infection and cancer. Macrophages carry out their microbicidal and tumoricidal activities by the oxygen-dependent killing via products of oxidative metabolism such as hydrogen peroxide, superoxide anion, and hydroxyl radical, and the oxygen-independent killing via cytokines and hydrolytic enzymes. We previously reported that PYC inhibited generation of reactive oxygen species such as peroxides in macrophages (Bayeta and Lau, 2000) indicating its ability to abolish the oxygen-dependent killing mechanism. This inhibition is used to explain the anti-inflammatory property of PYC reported in the literature (Blazso *et al.*, 1994; Blazso *et al.*, 1997). Our curiosity to find out whether or not PYC affected the oxygen-independent killing mechanism prompted us to investigate the effects of PYC on macrophage phagocytosis and cytokine secretion. TNF-α and IL-1β were chosen for our investigation because these two cytokines play an important role in regulating normal immune functions. Data from this study indicate that PYC did not inhibit macrophage phagocytosis and secretion of TNF-α and IL-1β. On the contrary, incubation of macrophages with PYC resulted in a greater phagocytic activity and increased levels of TNF-α and IL-1β. Thus, even though our previous study showed PYC to cripple oxygen-dependent killing mechanism, it is intriguing that the present study demonstrates PYC's ability to enhance phagocytosis and cytokine secretion—a part of the oxygen-independent killing mechanism of macrophages.

An increase in cell size is one of the earliest characteristics observed in activated macrophages and is related to the degree and rate of cell spreading; activated cells spread more rapidly and to a greater extent than the resident cells (Adams and Hamilton, 1984; Enane *et al.*, 1993). We observed a significant increase in cell size with PYC indicating its ability to activate macrophages. Activated macrophages exhibit a greater phagocytic activity than the resident cells (Zelikoff *et al.*, 1991). In our study, PYC-treated macrophages indeed exhibited a greater phagocytic capability than the untreated cells. With as little as 8.75 µg/ml of PYC, the phagocytosis increased by 27% as compared with the untreated control cells (Fig. 1). Activated macrophages are known to increase their secretion of hydrolytic enzymes and cytokines. We did not investigate hydrolytic enzymes in the present study. Previous studies have, however, demonstrated PYC's ability to enhance the activity of enzymes in endothelial cells and

macrophages (Wei *et al.*, 1997; Bayeta and Lau, 2000). The present study shows a significant increase of TNF-α and IL-1β secretion when macrophages were incubated with PYC. These two cytokines are unique in that they enhance their own release as well as that of each other (Durum and Oppenheim, 1989). In addition, they provide costimulatory signals to enhance the activation of helper T- lymphocytes and thus promote both the humoral (B-lymphocytes) and the cell-mediated (T-lymphocytes) immune responses (Oppenheim and Ruscetti, 1997).
Several studies have demonstrated that PYC is a potent anti-inflammatory phytochemical (Blazso *et al.*, 1994; Blazso *et al.*, 1997; Bayeta and Lau, 2000). PYC differs from currently used steroidal or non-steroidal anti-inflammatory drugs in that it does not exhibit any adverse side-effects (Bayeta and Lau, 2000). Unlike most anti-inflammatory drugs that exert immunosuppressant activity against B- and T-lymphocytes, the present study shows PYC's ability to increase cytokines known to promote immune functions. Cheshier *et al.* (1996) used retro virus and chronic ethanol intoxication to induce immunosuppression in mice. Feeding with PYC delayed the development of immune dysfunction in this animal model. PYC restored the imbalanced cytokine secretion by T-helper 1 and T-helper 2 cells, which are important in cellular and humoral immunity, respectively. In our study with senescence-accelerated mice, we demonstrated that PYC can restore the immune and hemopoietic functions that are altered in the aging process. Feeding animals with PYC significantly improved the severely depressed B- and T-lymphocyte responses to mitogens (Liu *et al.*, 1999). These studies suggest that PYC can enhance B and T cell function and at the same time it can exert potent anti-inflammatory effects.
In conclusion, the data from this *in vitro* study indicate that PYC is capable of activating macrophages to enhance phagocytosis and the secretion of TNF-α and IL-1β. These results suggest that PYC may play an important role in modulating the immunological function by means of macrophage activation. PYC has been shown to have potent anti-inflammatory property. Unlike anti-inflammatory drugs currently in use, PYC is a novel and unique therapeutic agent in that it does not exhibit immunosuppressant activity. Whether or not these beneficial effects occur in humans warrants further clinical studies.

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