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In Vitro and In Vivo Inhibition of LPS-Stimulated Tumor Necrosis Factor-α Secretion by the Gallotannin β-D-Pentagalloylglucose

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Abstract—The naturally occurring gallotannin β-D-pentagalloylglucose (β-PGG) decreases tumor necrosis factor-alpha (TNF-α) output from human peripheral blood mononucleocytes exposed to lipopolysaccharide (LPS) by as much as 90% (vs control) at ~5 μM concentration. A qualitatively similar but less pronounced effect (~50% decrease) was observed in the serum of rats dosed with both LPS and β-PGG. These results may have relevance to therapies that target disease states characterized by an overproduction of TNF-α.

Chronic overproduction of the cytokine tumor necrosis factor-α (TNF-α)[1,2] has been implicated as a significant mediator of a variety of inflammatory diseases including rheumatoid arthritis and Crohn's disease.[3] In addition, acute oversecretion of TNF-α [and interleukin-1β (IL-1β)] from peripheral blood mononucleocytes (PBMCs) as a consequence of exposure to the bacterial toxin lipopolysaccharide (LPS) contributes to the etiology of septic shock. The complex and multi-faceted cascade of events that initiates with LPS/PBMC receptor(s) association and concludes with TNF-α release into the serum offers many potential sites for therapeutic intervention of the septic shock response. Strategies designed to interrupt this response have focused, in turn, on (1) interfering with the triggering LPS/CD14 receptor interaction,[4,5] (2) inhibiting or downregulating various members of the signaling cascade that link the membrane-bound receptor(s) with transcriptional activators, (3) disrupting transcriptional regulation, (4) suppressing post-translational cleavage of the first-formed proTNF-α protein, and (5) sequestering serum soluble TNF-α with a variety of macromolecules (e.g., antibodies and soluble TNF-α receptors).[6,7] Each approach has generated guarded optimism based on promising in vitro results, but in vivo performance has been less satisfactory. In many instances, the nucleoside analogues and polyheterocycles examined as small molecule inhibitors of enzymes or receptors in the cytosolic signaling pathway don't exhibit sufficient specificity to recommend therapeutic use.[6,7] Herein, we report that a member of an entirely different class of small molecule agents, the simple gallotannin β-D-pentagalloylglucose (β-PGG, **1**), is an effective inhibitor of LPS-stimulated TNF-α secretion from human PBMCs. This observation may encourage further investigation of the largely nontoxic tannin[8–10] family of secondary plant metabolites as a source of leads for the development of novel treatments for septic shock and other TNF-α-mediated inflammatory diseases.

We have documented the immunostimulatory properties of the dimeric ellagitannins coriariin A (**2**) and agrimoniin (not shown), and the dimeric gallotannin–ellagitannin hybrid **3**.[11] Substantial increase in TNF-α secretion attended exposure of human PBMCs to each of these dimeric species, an observation that may relate to the in vivo antitumor activity observed for these ellagitannins by Okuda[12,13] and by Miyamoto.[14–16] In

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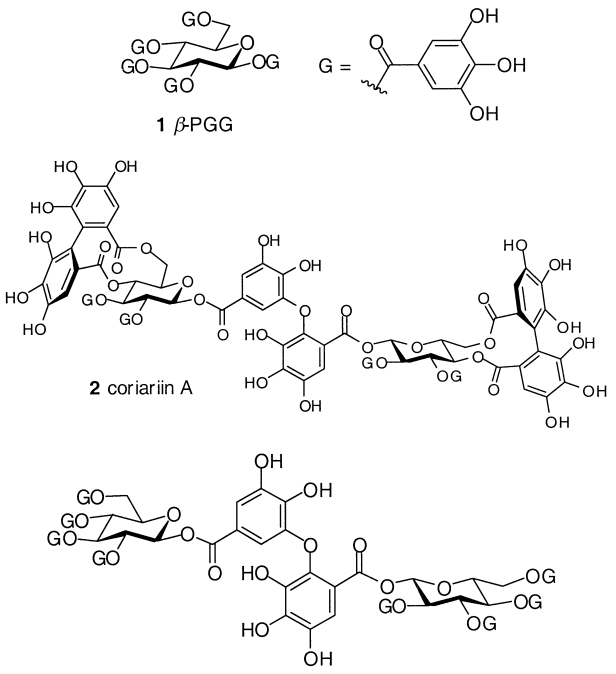

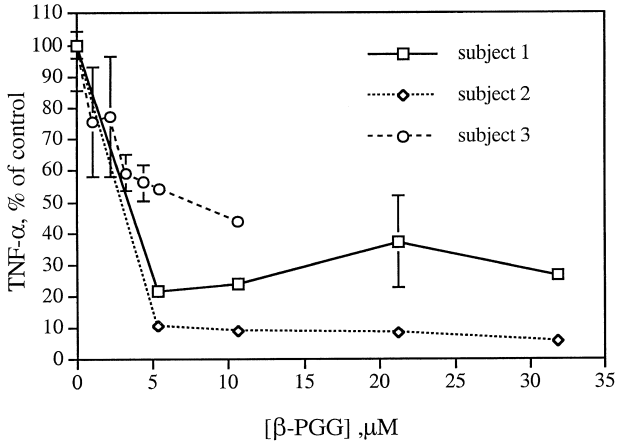

Figure 1. Dose–response data for TNF-α secretion from three different subjects' PBMCs upon exposure to 5 μg/mL of LPS and varying concentrations of β-PGG 1.

contrast, the monomeric gallotannin β-PGG **1** induced only very low levels of TNF-α production from human PBMCs at similar concentrations, a result again consistent with the earlier in vivo studies that indicated that **1** is not an effective antitumor agent.[12] The structural similarity between β-PGG and the active dimer **3**, in conjunction with the significant disparity in biological response [**1** (10 μM) → 275 pg/mL of TNF-α; **3** (10 μM) → 2160 pg/mL of TNF-α],[11] raised the possibility that **1** may act as an antagonist of **3**. This hypothesis was explored with PBMCs harvested from three subjects (Fig. 1). The PBMCs were stimulated with a fixed concentration (5 μg/mL) of LPS, followed by treatment with β-PGG[17] in the concentration ranges shown. ELISA analysis of the cell culture supernatants after a 4 h incubation revealed markedly lower TNF-α levels compared to control (PBMC + LPS).[18] This time period was chosen based on earlier observations of the time course of cytokine secretion from tannin-stimulated PBMCs.[11,14] To compensate for the inherent variability between cells from different subjects, the TNF-α secretion data were normalized to 100% response for the control. All three subjects showed pronounced suppression of LPS-stimulated TNF-α release upon β-PGG treatment. Maximum inhibition of TNF-α output was achieved at an applied β-PGG concentration of approximately 5 μM for each subject. Beyond that point, the effect leveled off up to ~53 μM of β-PGG (not shown). Examination of the 1–5 μM β-PGG concentration regime with subject 3 indicated that TNF-α secretion scaled inversely with tannin concentration over this range. The maximal inhibition of TNF-α secretion varied significantly between the three subjects, with as much as a 95% decrease versus control observed with subject 2 at higher β-PGG concentrations. A time dependence for this inhibitory activity was evident, as no reduction of secreted TNF-α levels resulted after incubation of the PBMCs with LPS and **1** for 24 h.

Extension of these inhibition studies to an in vivo rat model system was investigated next (Figs. 2 and 3). In all cases, the compound of interest was administered intravenously to chronically catheterized, conscious, unrestrained rats (250 g each). Initial toxicity studies indicated that a 50–60 mg/rat dose of β-PGG infused over 30 min led to a precipitous and lethal drop in blood pressure, whereas a 30 mg/rat dose (30 min) had no detectable effect on blood pressure or blood glucose levels, and a 4 mg dose with LPS-stimulated rats had no effect on TNF-α levels. Consequently, a dose of ~17 mg of **1** per rat was employed in subsequent experiments. This dose of β-PGG was administered to rats 6–9 as an initial 10 mg charge followed by continuous infusion of the

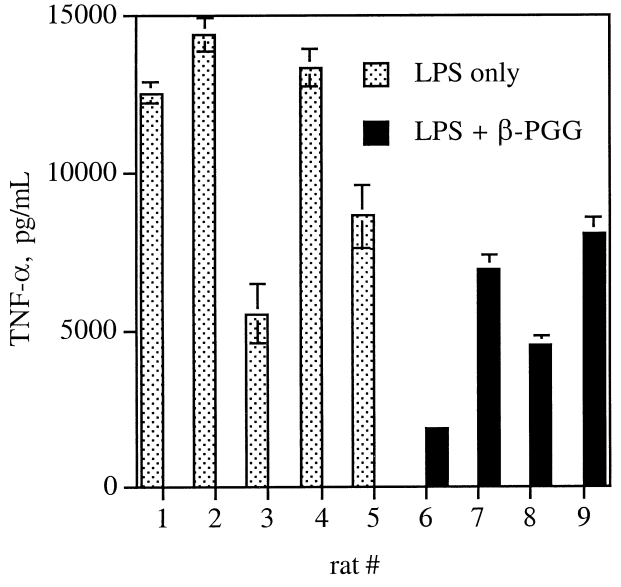

Figure 2. Plasma TNF-α levels at 90 min from rats treated with either LPS only (0.25 mg/rat, Nos. 1–5) or LPS (0.25 mg/rat, Nos. 6–9) and β-PGG **1** (17 mg/rat, Nos. 6–9).

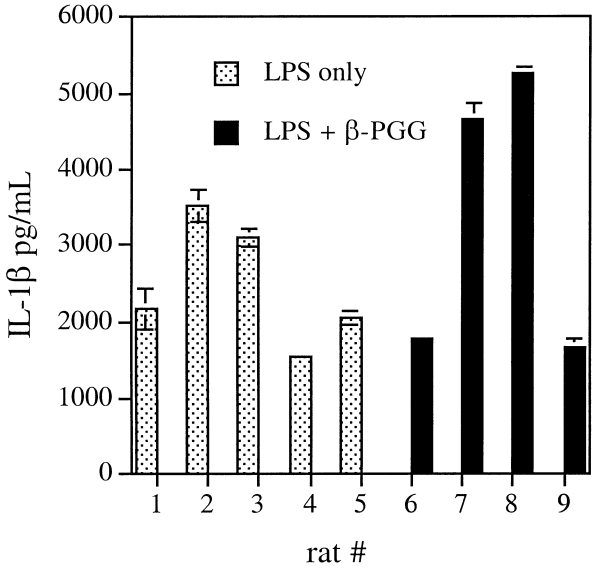

Figure 3. Plasma IL-1β levels at 90 min from rats treated with either LPS only (0.25 mg/rat, Nos. 1–5) or LPS (0.25 mg/rat, Nos. 6–9) and β-PGG **1** (17 mg/rat, Nos. 6–9).

remaining ~7 mg over 90 min, whereas rats 1–5 received only saline. All animals were treated with 0.25 mg of LPS 10 min after the start of β-PGG addition. Blood plasma samples were collected at 90 min and assayed by ELISA for TNF-α and IL-1β levels. The data indicate that, on average, the rats given β-PGG had about half of the circulating TNF-α levels as did the untreated rats. Despite this decrease in serum TNF-α level, the rats dosed with **1** showed no amelioration of the physiological symptoms characteristic of septic shock [e.g., blood glucose levels in mg/dL at 90 min: control, 55 (±5), LPS, 155 (±19), LPS + β-PGG, 170 (±13)]. It is possible that the sustained septic shock response stems from an enhanced production of the allied cytokine IL-1β upon stimulation by both LPS and β-PGG (Fig. 3). Earlier work illustrated that β-PGG itself induces substantial secretion of IL-1β from hPBMCs.[11]

These preliminary results demonstrate that a member of the tannin family of secondary plant metabolites, β-D-pentagalloylglucose (**1**), is an effective immunoregulator of the LPS-mediated TNF-α response in both human PBMCs and in live rats. The mechanism(s) by which this heretofore unrecognized inhibitor operates remains a matter of speculation at present, but direct antagonism of LPS cannot be dismissed. Further work to design more selective and more active tannin-inspired agents for inhibition of the LPS-stimulated TNF-α response is in progress.

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18. Representative procedure: Human PBMC Inhibition Data. Fresh heparinized blood was obtained from healthy human subjects (ages 20–34). PBMCs were isolated using reported procedures[14] (see also Kanof, M. J.; Smith, P. D. I. In *Current Protocols in Immunology*; Coligan, J. E., Kruisbeck, A. M., Margulies, D. M., Eds.; New York: Wiley Interscience, 1991; Vol. 1, Unit 7.1). The cells were counted and the viability was determined by Trypan Blue exclusion (typically, viability exceeded 95%). 0.5 mL samples of PBMC suspension (2×10^6 cells/mL in RPMI 1640) were stimulated with 5 μg/mL of LPS in a 5% CO_2, 37 °C humidified incubator for 45 min. At this time, the non-control wells were treated with the appropriate amount of a β-PGG stock solution in Hanks' Balanced Salt Solution to furnish the desired concentration range. Each concentration value was run in triplicate, and blank runs ensured that (bacterial) contamination did not complicate the experiments. After 4 h, culture supernatants were harvested and analyzed for TNF-α by Enzyme Linked Immunosorbent Assay (ELISA) using kits purchased from R&D Systems, Minneapolis, MN. The cytokine values reported are mean ± SEM.