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IMMUNOSTIMULATION BY PLANT POLYPHENOLS: A RELATIONSHIP BETWEEN TUMOR NECROSIS FACTOR-α PRODUCTION AND TANNIN STRUCTURE

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Abstract: The ability of the naturally occurring tumoricidal ellagitannin coriariin A to stimulate secretion of both IL-1β and TNF-α from human peripheral blood mononuclear cells has been demonstrated. Companion studies with the monomeric gallotannin β-D-pentagalloylglucose and a synthesized dimeric gallotannin-ellagitannin hybrid suggest that TNF-α rather than IL-1β is the causative agent in tannin-mediated antitumor action in vivo, in contrast to an earlier proposal.

The ellagitannin family of plant polyphenols spans a class of over 500 structurally diverse members.[1-4] An increasing interest in the role played by these secondary plant metabolites in tannin-rich folk medicines from China and Japan has led to the identification of several ellagitannins which display high levels of activity in anticancer and antiviral assays. These ellagitannins typically exhibit inherently low cytotoxicity and thus may serve as promising leads for the development of novel therapeutics.[3,5,6] An examination of the in vivo antitumor potency of several ellagitannins has shown that the dimeric ellagitannins are more potent tumoricidal agents compared to the monomeric (gallo)ellagitannins.[7,8] The intraperitoneal administration of tannins to mice before or after sarcoma-180 tumor inoculation elicits the same extent of tumor regression.[9,10] These observations suggest that endogenously inducible factors may play a role in the antitumor activity of the ellagitannins. Miyamoto's in vitro studies on the molecular basis for the antitumor activity of a range of tannins revealed their ability to stimulate the secretion of interleukin-1β (IL-1β) from both mouse peritoneal exudate cells and human peripheral blood mononuclear cells (h-PBMC's).[8] IL-1β is capable of upregulating the activity of tumoricidal natural killer cells,[11,12] prompting the Hokuriku group's suggestion that this cytokine mediates the in vivo antitumor activity of the ellagitannins.[8,10] However, IL-1β's pleitotropic nature, systemic toxicity and relatively late entry into the cytokine cascade[13] all suggest that other immune response elements may be involved. In particular, the cytokine tumor necrosis factor-α (TNF-α) is an attractive candidate for immunomediated tumor remission. While there is no antitumor activity directly attributable to IL-1β, there is evidence implicating TNF-α as the direct causative agent in tumor cell death.[14] In addition, TNF-α itself can induce the production of IL-1β from h-PBMC's both in vivo and in vitro.[15] Thus, if tannins do stimulate TNF-α secretion, Miyamoto's measurement of IL-1β levels actually may have reflected at least some secondary production attributable to nascent TNF-α. In order to test the premise that TNF-α actually mediates tannin-induced tumor remission, the ability of a documented tumoricidal

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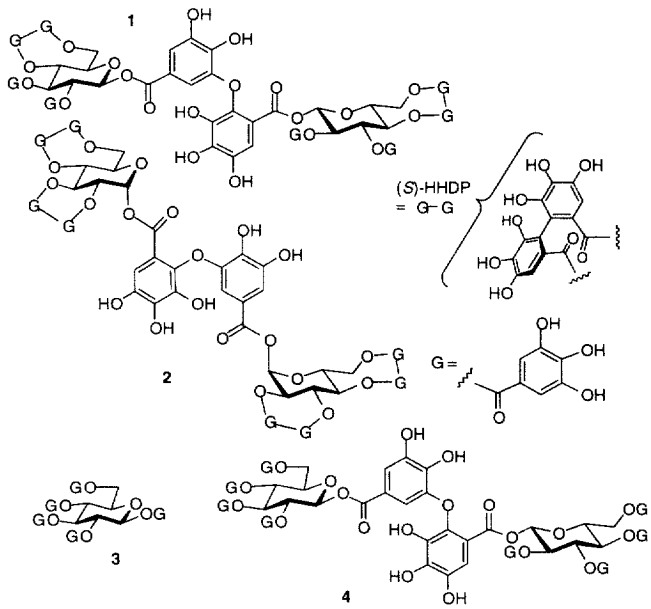
ellagitannin, as well as a representative gallotannin and a synthesized gallotannin-ellagitannin hybrid, to induce secretion of this cytokine was explored.

The dimeric antitumor ellagitannins coriariin A (**1**) and agrimoniin (**2**),[16] the monomeric gallotannin β-D-pentagalloylglucose (β-D-PGG) (**3**)[17] and the dimeric "gallotannin" **4**[18] were all examined in this study. Compound **4** is an analog of coriariin A and is identical to the parent ellagitannin except that the galloyl rings at O(4) and O(6) are not joined in a hexahydroxydiphenoyl (HHDP) linkage. h-PBMC's were incubated with the compound under investigation for the indicated length of time and the amounts of both IL-1β and, independently, TNF-α, present in the culture supernatants was determined using commercially available ELISA kits.[19] In each case, LPS was used as a positive control while untreated cells were reserved as negative controls.



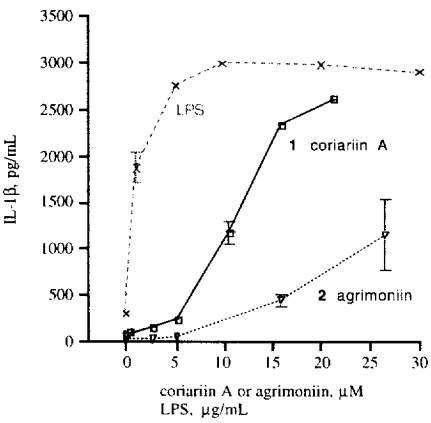


Figure 1. Il-1β release from h-PBMC's upon 4 h exposure to varying concentrations of coriariin A (1), agrimoniin (2), and LPS.

Initial trials with the natural products **1** and **2** were pursued under conditions described by Miyamoto.[10] After a 4 h treatment with 28 μM agrimoniin (**2**), h-PBMC's secreted ca. 1 ng/mL of IL-1β above blank (Fig. 1), a comparable result to that reported earlier (ca. 1.2 ng/mL at 20 μM agrimoniin).[8] Using these same experimental conditions to facilitate comparison with the prior studies from Hokuriku University, coriariin A (**1**) also demonstrated significant IL-1β inducing ability, perhaps twice that of agrimoniin on a molar basis (Fig. 1). Additional scouting experiments using a qualitative L929 murine fibroblast lysis assay[20,21] with both **1** and **2** revealed that these dimeric ellagitannins also promoted release of TNF-α in substantial amounts. This indirect measure of TNF-α levels in the h-PBMC supernatant provided the first evidence which implicated this cytokine in tannin-mediated antitumor activity and encouraged a more quantitative assessment of the TNF-α liberating capacity of **2** and the related species **3** and **4** as described below.

The time course for agrimoniin-mediated IL-1β secretion from h-PBMC's was delineated by Miyamoto.[10] Significant release of this cytokine was evident after a 4 h treatment, with maximal secretion at ca. 24 h. In

contrast, the kinetics of both IL-1β and TNF-α release from the h-PBMC's used in this study upon treatment with **3** or **4** followed measurably different profiles (Figs. 2 and 3). In all cases, a maximum value of cytokine discharge was observed at ca. 24 h (similar to Miyamoto's system), but only trace amounts of either cytokine were detected at the earlier 4 h reading. However, these data clearly demonstrate that both **4** and to a larger extent **3** are effective at stimulating the production of IL-1β from h-PBMC's. In comparison, the dimeric structure **4** appears to be much more proficient than the lower homolog **3** at eliciting TNF-α release at these particular concentration values. These encouraging observations prompted a more detailed exploration of the dose-response profile for the polyphenolic constructs **1**, **3**, and **4**. Unfortunately, a limited supply of the natural product agrimoniin precluded examination of its TNF-α response.

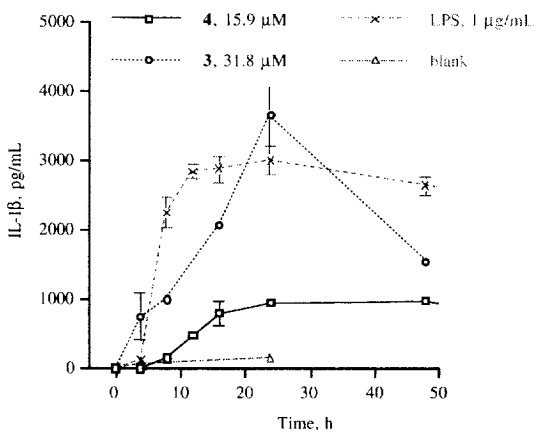


Figure 2. Time course of IL-1β release from h-PBMC's stimulated by fixed concentrations of β-D-PGG (3), 4, and LPS.

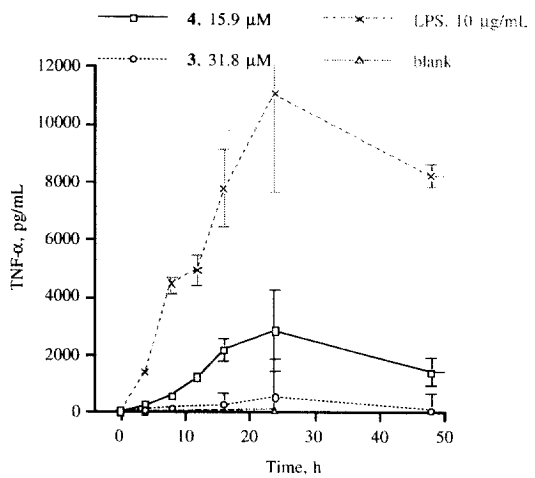


Figure 3. Time course of TNF-α release from h-PBMC's stimulated by fixed concentrations of β-D-PGG (**3**), **4**, and LPS.

h-PBMC's from two different subjects were utilized in independent experiments to obtain both IL-1β and TNF-α dose-response curves for the monomeric species **3** and its dimeric counterpart **4** at 24 h exposure, Figs. 4–7. The coriariin A and LPS data are included in these graphs for comparison purposes. Figs. 4 and 5 illustrate that β-D-pentagalloylglucose (**3**) is similar to coriariin A in its ability to induce IL-1β secretion in either subject. In addition, these graphs reveal that similar properties also attend the *O*-1-galloyl coupled dimer of **3**, gallotannin-ellagitannin hybrid **4**. Taken together, these results provide no support for the proposition that these polyphenolics must meet stringent molecular recognition criteria to initiate IL-1β release. Since Miyamoto's mouse studies did in fact discern well-defined structural requirements for anti-tumor activity among tannins (i.e., life span increase: **1** (238%), **3** (82%); regressors: **1** (3/6), **3** (0/6)), the role of IL-1β in the whole animal antitumor response is called into question.

The TNF-α release data (Figs. 6 and 7), however, tell a different story. β-D-PGG (**3**) promotes much less TNF-α discharge at comparable concentrations in either subject than does the dimer **4** or coriariin A (**1**). In

addition, the analog **4**'s dose-response profile is similar to that of coriariin A, at least in the lower concentration range. These data suggest that there is a structure-based discrimination between the monomeric and dimeric tannin species by some recognition element in the TNF-α induction pathway within h-PBMC's. This difference in TNF-α eliciting ability parallels the overall trend in antitumor properties for the mouse/sarcoma-180 system. Moreover, the similarity in IL-1β secretion profiles among the dissimilar species **1**, **3**, and **4** plausibly may just be an artifact of the first-formed TNF-α's ability to upregulate IL-1β production.

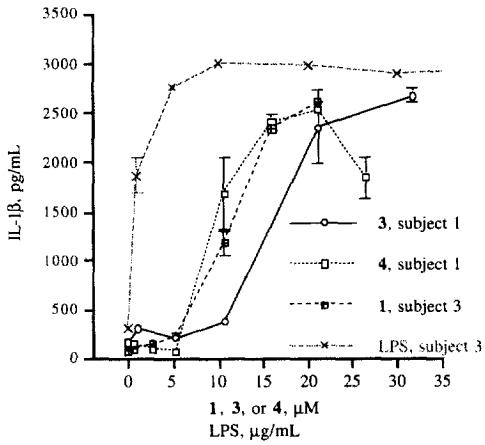


Figure 4. Il-1β release from subject 1's h-PBMC's upon exposure to varying concentrations of β-D-PGG (**3**) (24 h), the dimeric gallotannin **4** (24 h), coriariin A (**1**) (4 h), and LPS (4 h).

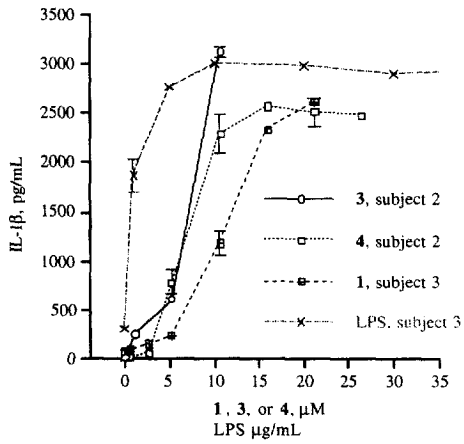


Figure 5. Il-1β release from subject 2's h-PBMC's upon exposure to varying concentrations of β-D-PGG (**3**) (24 h), the dimeric gallotannin **4** (24 h), coriariin A (**1**) (4 h), and LPS (4 h).

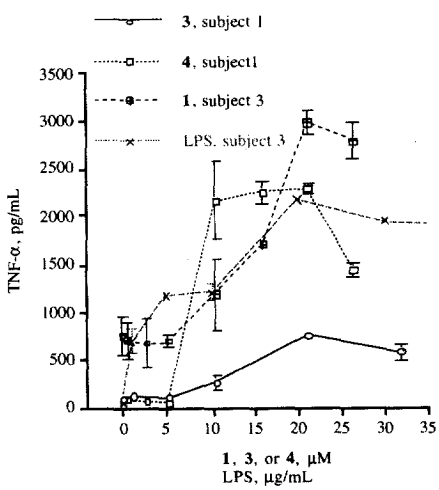


Figure 6. TNF-α release from subject 1's h-PBMC's upon exposure to varying concentrations of β-D-PGG (**3**) (24 h), the dimeric gallotannin **4** (24 h), coriariin A (**1**) (4 h), and LPS (4 h).

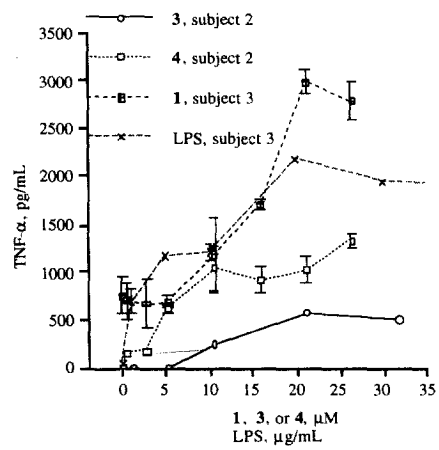


Figure 7. TNF-α release from subject 2's h-PBMC's upon exposure to varying concentrations of β-D-PGG (**3**) (24 h), the dimeric gallotannin **4** (24 h), coriariin A (**1**) (4 h), and LPS (4 h).

In conclusion, the ability of the dimeric tannin coriariin A (**1**) and the coriariin A analog **4** to induce higher levels of TNF-α from h-PBMC's compared to the monomeric compound β-PGG (**3**) has been demonstrated. This observation is in accord with earlier in vivo studies wherein the dimeric tannins demonstrated better antitumor activity when compared to the monomeric tannins. These experimental results suggest that the extent of TNF-α production from h-PBMC's incubated with tannins may correlate with the antitumor potency of tannins. The activity of the dimeric gallotannin **4** was indistinguishable from the activity of the related ellagitannin coriariin A, raising the possibility of using this structurally simpler, synthetically accessible species as a lead in the development of tannin-based chemotherapeutic agents.

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19. Representative procedures follow: Human IL-1β and TNF-α Enzyme Linked ImmunoSorbent Assay

(ELISA) kits were purchased from R and D Systems, Minneapolis, MN. Fresh heparinized blood was obtained from healthy human subjects (ages 20-34).

Dose-Response Data: General Procedure. h-PBMC's were isolated by reported procedures[10] (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%). The concentration of the cells in the 0.5 mL wells was adjusted to $1x10^6$ cells/well by diluting with the required amount of RPMI 1640.

The appropriate amount of a tannin (or LPS) stock solution in Hanks Buffer Saline Solution was added to each well to furnish the concentration values reported in the Figures. Each concentration value was run in triplicate, and blank runs ensured that (bacterial) contamination did not complicate the experiments. The culture plates were incubated in a 5% CO_2, 37 °C humidified incubator for the indicated time. At the end of the time interval, 450 μL of the culture supernatant from each well was harvested, after centrifugation at 400 g, 25 °C, 10 min, with brake, and stored at -78 °C pending ELISA analysis for the cytokine(s). The ELISA assays were conducted per the manufacturer's instructions using standard calibration curves to calculate cytokine concentration from observed absorbance readings. The cytokine values reported are averages of three runs ± SE.

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