PADMA-28, A Tibetan herbal preparation is an inhibitor of inflammatory cytokine production

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ABSTRACT. ***Background:*** **Previous studies have shown that PADMA-28, a multicomponent, traditional Tibetan herbal plant preparation possesses a variety of beneficial effects on several experimental models of inflammatory and immune processes, including autoimmune diabetes and autoimmune encephalomyelitis. In humans, PADMA-28 attenuated the symptoms associated with intermittent claudications in atherosclerotic patients. Objective: To assess the effect of PADMA 28 on the immune system, e.g. cytokine (interleukins) production.** ***Design:*** **Cytokine production by human blood monocytes (derived from12 healthy donors) stimulated *in vitro*, either by endotoxin (LPS) from *Salmonella typhi* or by lipoteichoic acid (LTA) from group A Streptococci was modulated by PADMA-28.** ***Results:*** **The present study showed that an aqueous extract of PADMA-28 strongly decreased the production of the inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α, and more moderately, also decreased the anti-inflammatory cytokine IL-10 induced by LPS. However, the LTA - induced IL-10 production was [not significantly] increased by the low dose PADMA-28, while not effected at all by the higher dose of PADMA-28.** ***Conclusions:*** **The data from these finding suggest a possible clinical efficacy of PADMA-28 either in autoimmune and in inflammatory conditions or in post-inflammatory sequelae, as previously shown in *in vivo* and human studies, probably by decreasing inflammatory cytokines.**

Keywords: PADMA-28, herbal medicine, inflammatory cytokines

INTRODUCTION

Recently, there has been worldwide interest in the role of medicinal botanicals in complementary medicine [1]. Previous studies have shown that PADMA-28, a traditional Tibetan herbal preparation, which is comprised of 20 different plants, possesses a variety of beneficial effects on inflammatory and immune processes. Aqueous extracts derived from this herbal preparation were found to markedly inhibit chemotaxis [2], to possess anti-oxidant and anti-proteinase activities [3, 4] and to inhibit inducible nitric oxide synthesis in a macrophage cell line [5]. Also, aqueous and ethanolic extracts of this multicomponent formula exhibited antimicrobial properties on Gram-positive bacteria as well as Gram-negative *Klebsiella pneumoniae*, comparable to five other European herbs used for skin infections [6]. Further, it was shown to inhibit oxidative burst in human neutrophils, neutrophil elastase, the peroxidation of lipids as well as the killing of epithelial and endothelial cells in cultures treated by oxidants [7]. PADMA-28 was also found to decrease the oxidative burst responses of monocytes and to improve fibrinolysis in patients with stable intermittent claudication [8], to attenuate intermittent claudication in patients [9-14] and the potentials of the electroretinogram in lipid metabolism and vascular changes [15]. More recently, PADMA-28 was also found to significantly inhibit the development of type I diabetes in autoimmune NOD mice (Weiss, Barak, Raz, Ginsburg, to be published), and also to delay the development of allergic encephalomyelitis in SJL mice (Raibstein, Weiss, Ginsburg and Barak, to be published). These findings strongly suggest that PADMA-28 might also prove to be an effective agent as a modulator of cytokine-dependent autoimmune phenomena in clinical settings.

Inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) have been shown to be main players in the induction and maintenance phase of the inflammatory process [16]. These cytokines have an important role in the pathogenesis of a variety of acute and chronic diseases. For example, disorders such as local and systemic infections [16], septic shock [17], rheumatoid arthritis [16] autoimmune diseases such as nephritis, vasculitis, inflammatory bowel disease [18] as well as leukemias [19], might all be mediated by proinflammatory cytokines and their receptors [20].

The aim of the present study was to determine the modulating capacity of the herbal preparation PADMA-28 on the *in vitro* production of inflammatory and anti-inflammatory cytokines by human monocytes from healthy individuals, stimulated either by the key pro-inflammatory toxins, lipopolysaccharide (LPS) or by lipoteichoic acid (LTA). Both agents have been shown to act as main triggers of post-infectious sequelae [21, 22]. The

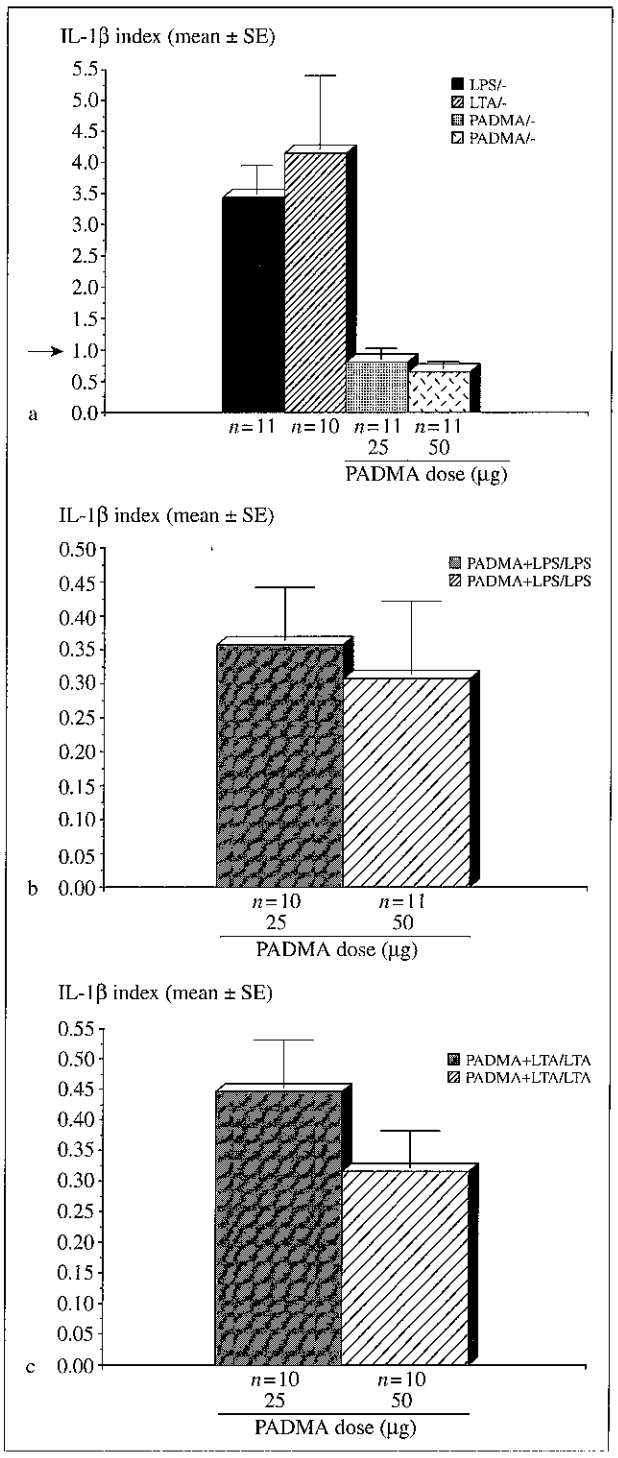

Figure 1
IL-1β production (pg/mL) by monocytes stimulated by a combination of LPS (**b**) or LTA (**c**) with PADMA-28 (25 and 50 μg) or stimulation by the individual LPS, LTA and PADMA (**a**).

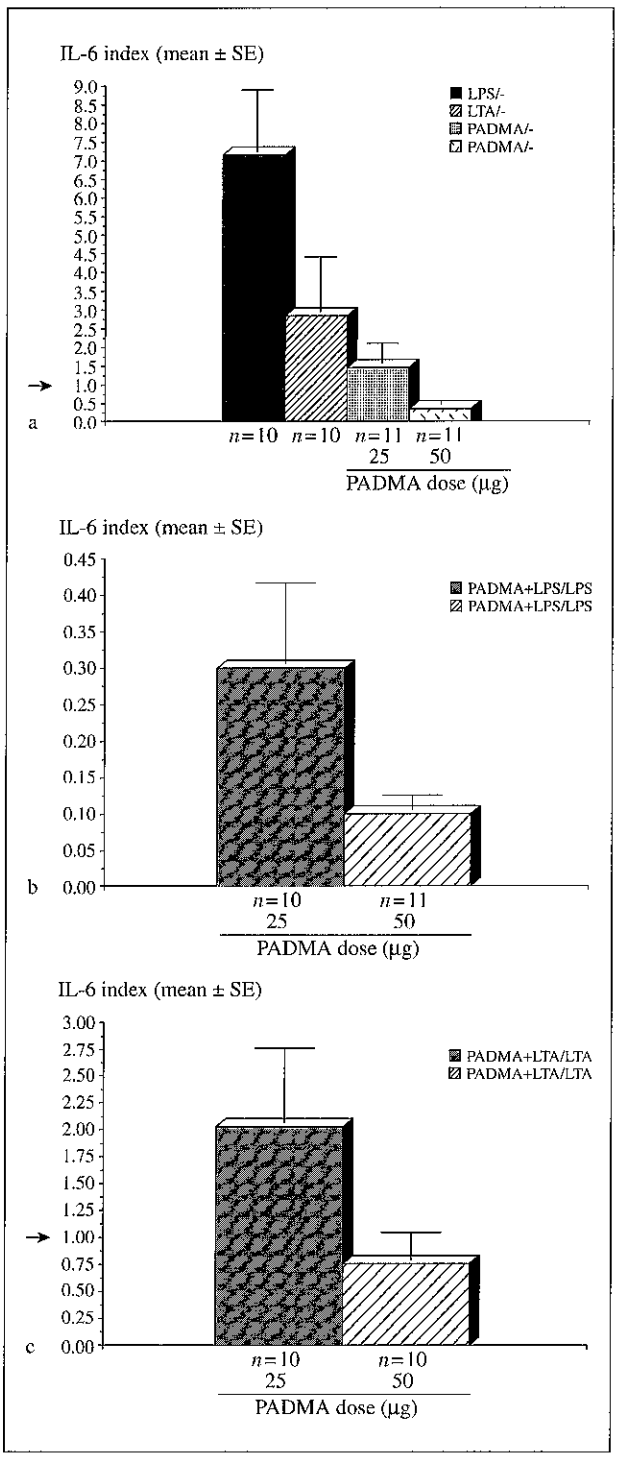

Figure 2
IL-6 production (pg/mL) by monocytes stimulated by a combination of LPS (**b**) or LTA (**c**) with PADMA-28 (25 and 50 μg) or stimulation by the individual LPS, LTA and PADMA (**a**).

trials in humans [9-15], the findings that microgram quantities, can strongly inhibit the production of proinflammatory cytokines by human monocytes *(Figures 1-5)* is of clear significance. Since stimulation of untreated controls with PADMA 28 did not induce any increase in IL-1β, IL-6 and IL-8 production (only a rise in TNF-α, by the low dose of 25 μg/mL of PADMA 28), it is unlikely that this herbal preparation was significantly contaminated with either LPS or LTA. Furthermore, our PADMA-28 preparations were cultured and shown to be sterile. It might also be speculated that since the anti-inflammatory cytokine effects of PADMA-28 are not due to a single agent, but rather to a combination of several agents, this herbal preparation could prove a valuable clinical tool to control and prevent the multiplicity of cytokine and other mediator cascades, generated in inflammatory and autoimmune insults [16-18].

Although the mechanisms by which PADMA-28 alters cytokine production is still not fully understood [2, 4, 5, 7], it is well established that both LPS and LTA interact with

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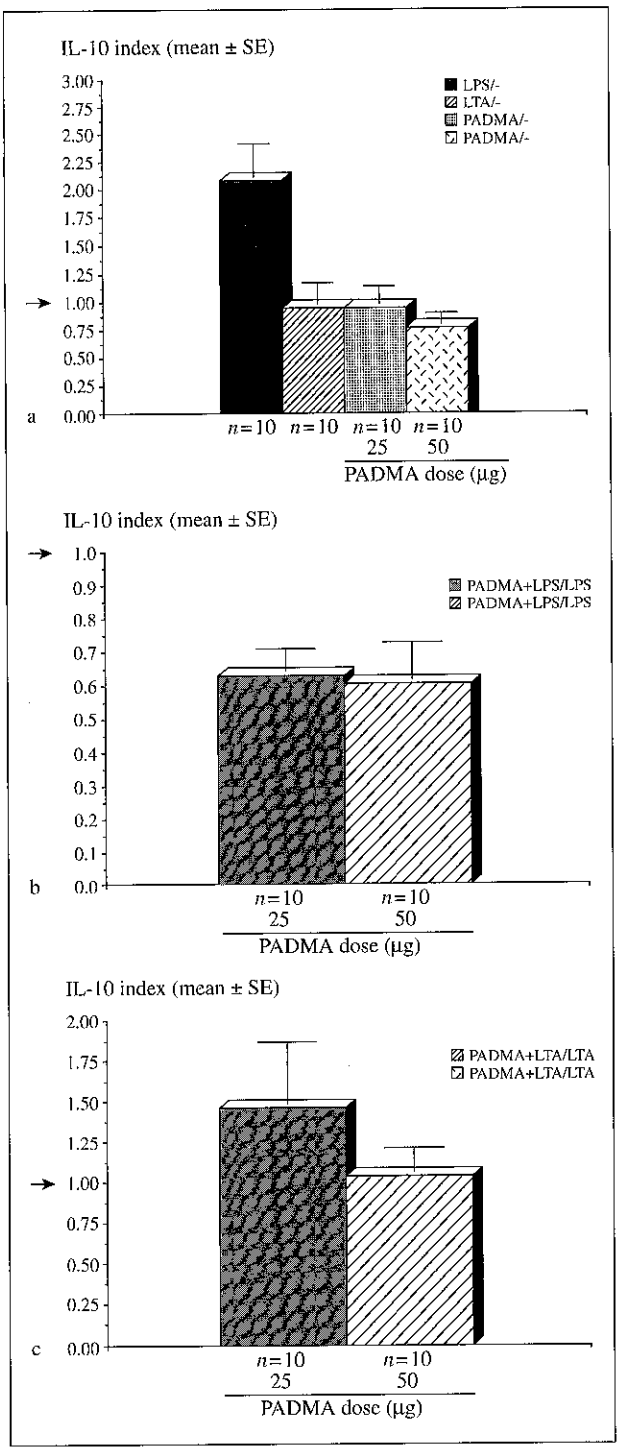

Figure 5
IL-10 production (pg/mL) by monocytes stimulated by a combination of LPS (**b**) or LTA (**c**) with PADMA-28 (25 and 50 µg) or stimulation by the individual LPS, LTA and PADMA (**a**}).

of interest that *in vivo*, PADMA-28 inhibited autoimmune diabetes in NOD mice (Ginsburg, Weiss, Barak, Raz in preparation.), and delayed the development of allergic encephalomyelitis in SJL mice immunized with antigens derived from the central nervous system (Raibstein, Ginsburg, Weiss, Barak, in preparation). Both these autoimmune phenomena are probably caused by an imbalance of Th1/Th2 cytokine production [31] and the reduction in inflammatory cytokine production shown in this study might account for the beneficial effect of PADMA-28. The data presented here are in accordance with previous observations that had suggested that PADMA-28 has the capacity to modulate several important mediators of inflammation, likely to be generated *in vivo* e.g. local and systemic inflammatory cytokine effects. Most probably, these effects, shown previously in animal models as well as in humans [6-15], are based on and can be also explained by our results here, namely, the significant reduction in inflammatory cytokine production following LPS and LTA induction, characteristic of inflammatory conditions.

Other herbal preparations have been shown previously by us and others to affect the immune system, as well as inflammatory responses. The most investigated preparations are Echinacea [32] and Sambucol (elderberry) [25, 26]. Echinacea, tested separately in our assay, reduced only moderately the production of inflammatory cytokines [26], similar to another study [33]. However, unlike PADMA-28, which, alone, had failed to directly stimulate inflammatory cytokine production (see above), Sambucol which also possesses potent anti-oxidant and antiviral activities [34], had a strong stimulatory effect on the production of both inflammatory and anti-inflammatory cytokines [25, 26], Therefore, the experiments with Sambucol probably necessitates an examination of its possible LPS and LTA content.

Taken together, since it is well established, that cytokines [28], reactive oxygen and nitrogen species, proteinases [3-6] and autoimmune phenomena are all directly involved in tissue damage during inflammation, infection and in post-infectious sequelea [6, 35, 36], the suppression of these proinflammatory activities by a non-toxic hebal preparation, such as PADMA-28, is of great clinical significance. Further work along these lines are now in progress.

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removed by aspiration, and the wells were washed three times with PBS. The adherent cells were cultured for 24 hours in RPMI medium in the absence of serum. PADMA 28 formulation (25/50 µg/well), LTA (10 µg/mL) or LPS (100 ng/mL) as control monocyte stimulators, were added to the monocytes. At the end of the culture period, (24 hours) the supernatants were harvested, centrifuged at 300 g for 10 min and stored at – 70 °C until assayed for cytokines, as has been previously reported [25, 26].

Cytokine assays

The levels (pg/mL) of the inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α and of the anti-inflammatory cytokine IL-10 were measured in supernatants of monocyte cultures, by a solid phase ELISA (R&D, Minneapolis, MN, USA). This assay employs a quantitative "sandwich" enzyme immunoassay technique. A monoclonal antibody specific for the cytokine molecule, was precoated onto the polystyrene microtiter plate. Standards and samples were introduced into the wells where the immobilized specific antibodies bind the cytokines. After washing away unbound proteins, the second enzyme-linked polyclonal or monoclonal antibody specific for the cytokine was added to the wells to "sandwich" the cytokine immobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells, causing the development of a color that was proportional to the amount of cytokine bound in the initial step. The color development was stopped by 2N sulfuric acid, and the intensity of the color was measured at 450 nm. A standard curve plotting the optical density versus the concentration of a given cytokine was prepared and used to determine the concentration of the cytokine in unknown samples, as previously reported [19, 20, 25, 26].

Stimulation index

Since the levels of cytokine production in all blood donors vary to a large extent, we have chosen to express the data for cytokine production as an index, rather than to express the results as pg/mL. The effect of the different formulations on the induction of inflammatory /anti-inflammatory cytokine production (pg/mL) was expressed as:

$$\text{Stimulation index} = \frac{\text{cytokine production with stimulant}}{\text{cytokine production without stimulant}} \quad \textit{Figure (a)}$$

$$\text{Stimulation index} = \frac{\text{cytokine production with PADMA}}{\text{cytokine production without PADMA}} \quad \textit{Figure (b, c)}$$

Statistical analysis

All statistical analyses were performed on the mean ± SE of individual indexes. Comparison between groups (unstimulated controls *versus* LPS - or LTA - stimulated cultures) was performed using the Mann-Whitney's test. A value of $P < 0.05$ was considered significant. Each group consisted of 12 donor cultures, two PADMA-28 concentrations ± LPS and ± LTA.

RESULTS

Effect of PADMA-28 on cytokine production

The production of the three inflammatory cytokines (IL-1β, IL-6, IL-8) was moderately decreased by the PADMA-28 - 50 µg/mL *(Figure 1a, 2a, 3a)*, as compared with the unstimulated control (index less than or around 1). Only basal production of TNF-α was increased (although a large SE was shown), by the low dose of PADMA-28 – 25 µg/mL, while the high dose – 50 µg/mL did not really have any effect *(Figure 4a)*.

PADMA-28 at both concentrations (25 or 50 µg/mL) significantly decreased the LPS - *(Figure b)* and LTA - *(Figure c)* induced IL-1β *(Figure 1)* and IL-8 *(Figure 3)*.

PADMA-28 (50 µg/mL) significantly reduced LPS-induced TNF-α *(Figure 4b)* and IL-6 production *(Figure 2b)*, as well as LTA-induced TNF-α *(Figure 4c)*.

LTA-induced IL-6 production *(Figure 2c)* and TNF-α production *(Figure 4c)* were increased by PADMA-28 - 25 µg/mL (although a large SE was seen).

In addition, PADMA-28 also significantly decreased the LPS-induced IL-10 production (the main anti-inflammatory cytokine) *(Figure 5b)*. LTA-induced IL-10 production was increased slightly (± SE) by 25 µg/mL PADMA-28, but was unaffected by the higher dose of PADMA-28 *(Figure 5c)*.

DISCUSSION

The results presented in this study show that PADMA-28, a versatile, non-toxic, herbal preparation shown to modulate the respiratory burst in human neutrophils, the peroxidation of lipids [6, 7], the generation of nitric oxide [3] and to attenuate intermittent claudication in atherosclerosis patients [9-15], is also a potent inhibitor of cytokine production induced in human monocytes by the two key proinflammatory agents, LPS and LTA *(Figures 1 to 5)*. These microbial-derived agents released primarily following bacteriolysis [27], are considered to be the main triggers of post-infectious sequelae and multiple organ failure resulting from infections by both Gram positive and Gram-negative bacteria [21], probably by inducing high levels of inflammatory cytokines [17].

PADMA-28 has been certified for OTC distribution, or as a food additive, in six European countries as well as in the USA (Padma Basic Inc). Since it is safe and has been shown to possess no apparent major side effects (mostly rare, mild gastrointestinal disorders), its ability to control inflammatory processes has been recommended for clinical use including for chronic inflammatory vascular disorder. The use of this herbal preparation might be especially important since scores of clinical trials of sepsis in humans, using single antagonists, have invariably failed to significantly prolong patients' lives [28].

Aqueous extracts from PADMA-28, are complex mixtures of herbs. In a previous publication, we have described the characterization of 31 compounds from PADMA-28, many of them for the first time [6, 7]. However, being a complex mixture of relatively low molecular weight substances, rich in bioflavonoids and in polyphenols, which lack toxicity to mammalian cells at concentrations up to 250 µg/mL, and which are also well tolerated in clinical

relevance of these findings to the elucidation of the mechanisms involved in tissue and organ damage in sepsis and septic shock, will be briefly discussed.

DONORS AND METHODS

Aqueous extract of PADMA-28

The herbal preparation PADMA-28 was kindly supplied in a powder form by the PADMA Company AG, Schwerzenbach, Switzerland [2, 6, 12]. In Switzerland, PADMA-28 was first registered in 1977 as an over-the-counter (OTC) medicinal product for therapeutic use in circulatory disorders.

This herbal preparation, used in Europe for more than 30 years, is a complex product consisting of 20 plants, natural camphor and calcium sulfate. The therapeutic effects of the preparation, which is made up of numerous chemical compounds, cannot be attributed either qualitatively or quantitatively to individual substances. The various constituents interact in an additive, synergistic and/or antagonistic manner, thus in this way, give the product its specific action profile. For more details on the the herbal preparations which make up one PADMA-28 tablet, the pharmacokinetics of this preparation and safety see PADMA AG's Investigator's Brochure, 2003.

Each tablet of PADMA-28 distributed OTC, contains 403 mg of the herbal mixture. It is comprised of: *Aegle sepiar fructus* (20 mg), Amomi Medic. fructus (25 mg), *Aquilgiae vulgaris herba* (15 mg), calcium sulfuricum pulv (20 mg), *Calendulae flos* (5 mg), Camphora Japon (4 mg), *Cardamomi fructus* (30 mg), *Caryophylli fructus* (12 mg), *Costi amari radix.* (40 mg), Hedychil rhizome (10 mg), *Lactucae sativa folium* (6 mg), *Lichen islandicus* (40 mg), *Liquiritiae radix* (15 mg), *Meliae tousend fructus* (35 mg), *Myrobalani fructus* (30 mg), *Plantaginis herba* (15 mg), *Polygoni avicularis herba* (15 mg), *Potentilae aureae herba* (15 mg), *Santali rubri lignum* (30 mg), *Sidae cordifoliae herba* (10 mg) Aconiti tuber (1 mg), and *Valerianae radix* (10 mg).

In addition to camphor and calcium sulfate, powdered PADMA-28 contains a mixture of gallotannins and cathechin tannins (1.4% w/w)% v/v, flavonoids (0.17% w/w), essential oils (1.2% V/m), saponins as glycyrrhizic acid, aconite (diester alkaloid), coumarin (0.32% vitamin K (0.2 μg/tablet), polysaccharides (inulin, starch lichenin, vegetable mucilages) silicates, and organic acids (isovaleric acid, malic acid, citric acid, oxalic acid and formic acid). Powdered PADMA - 28 extracted by organic solvents followed by chromatography on silica gel and GS-Mass spectroscopy, had yielded 31 components, which had been identified chemically [6]. Several components [6] had already been shown to possess anti-oxidant and anti-proteinase activities. However, due to technical difficulties, it was not possible to obtain any of these agents in sufficient quantities for use in the present study on cytokine production. Therefore, we chose to test the role of aqueous extracts from the whole, unfractionated powdered mixture of PADMA-28.

To prepare aqueous extracts, 403 mg portions of powdered PADMA-28, corresponding to one tablet, were vortexed for 15 min. at room temperature with 10 mL of sterile distilled water. The mixture was centrifuged at 3 000 *g* for 10 min. and the clear supernatant fluid was filtered through a Whatman no. 41 filter paper and kept at -30 °C. Lyophilization of such a preparation resulted in about 12% of the total PADMA-28 mixture. Microgram amounts of such a preparation possess potent anti-oxidant properties [5, 6]. To test for sterility, the filtered extracts were streaked on blood agar and on McConkey's medium (to isolate Gram negatives). Also, the anti-oxidant capacity of PADMA-28 extract was assayed by a recently developed, Luminol-dependent chemiluminescence technique. This involves a combination of sodium selenite, SIN-1 (morpholinosydnimine, a donor of both superoxide and NO), BSA and CO^{2+} (Ginsburg and Cohen, submitted for publication).

In the present study, PADMA-28 extracts containing 25 and 50 μg/mL were used to modulate cytokine production (see below).

Reagents

All the chemical reagents and preparations of lipopolysaccharide (LPS) from *Salmonella typhi* and lipoteichoic acid (LTA) from group A streptococci, were from Sigma Chemicals Co, St Louis MO, USA.

Since it had been stated that LTA preparations derived from commercial sources, might be contaminated by LPS [22], a sensitive, passive hemagglutination / hemolysis test [23] was used to test for the possible presence of LPS in the LTA preparation used. Briefly, human red blood cell suspensions were sensitized by LTA, in the presence of 10 units of crystalline trypsin to enhance binding of LTA, and washed in saline. The LTA-sensitized-RBC were then added to a twofold dilution of either anti-Salmonella or anti-Streptococcal sera, and in the presence of normal guinea pig serum as a source of complement. The anti-streptococcal antibody used was prepared by Dr. T.N Harris from the Children's Hospital, Philadelphia, by immunization of rabbits with an ammonium sulfate precipitation of supernatant fluids derived from cultures of Group A hemolytic streproccocci cultivated in a chemostat (steady state culture) and then lyophilized. While marked hemagglutination and hemolysis were induced with the anti-streptococcal sera, no such effect occurred with the anti-Salmonella serum, suggesting that the LPS preparation might not be significantly contaminated by LPS.

Blood monocyte cultures

Twelve donors (healthy students with no inflammatory conditions and currently not receiving any medication) participated in this study. They all submitted informed consent for their blood tests. Heparinized peripheral blood (50 mL) was obtained from each subject and diluted 1:1 with phosphate buffered saline (PBS). The mononuclear cells were separated by Ficoll-Hypaque sedimentation (400 *g*, 30 min) and washed three times with PBS (175 *g*, 10 min) to remove platelets. An aliquot of cells was counted in a hemocytometer and viability was checked using the trypan blue exclusion method. The adherent cells (> 90% monocytes) were identified by β-naphthyl acetate non-specific esterase staining [24]. The cells were suspended in RPMI 1 640 media, supplemented with 1 mM sodium pyruvate, 50 U/mL penicillin, 50 U/mL streptomycin, 2 mM L-glutamine, 1/100 MEM-vitamins and 2% inactivated human AB serum. The cells were plated in 24-well culture dishes at a concentration of 4×10^6 cells per well, and incubated for 90 min at 37 °C in a humidified atmosphere containing 5% CO_2. Non-adherent cells were

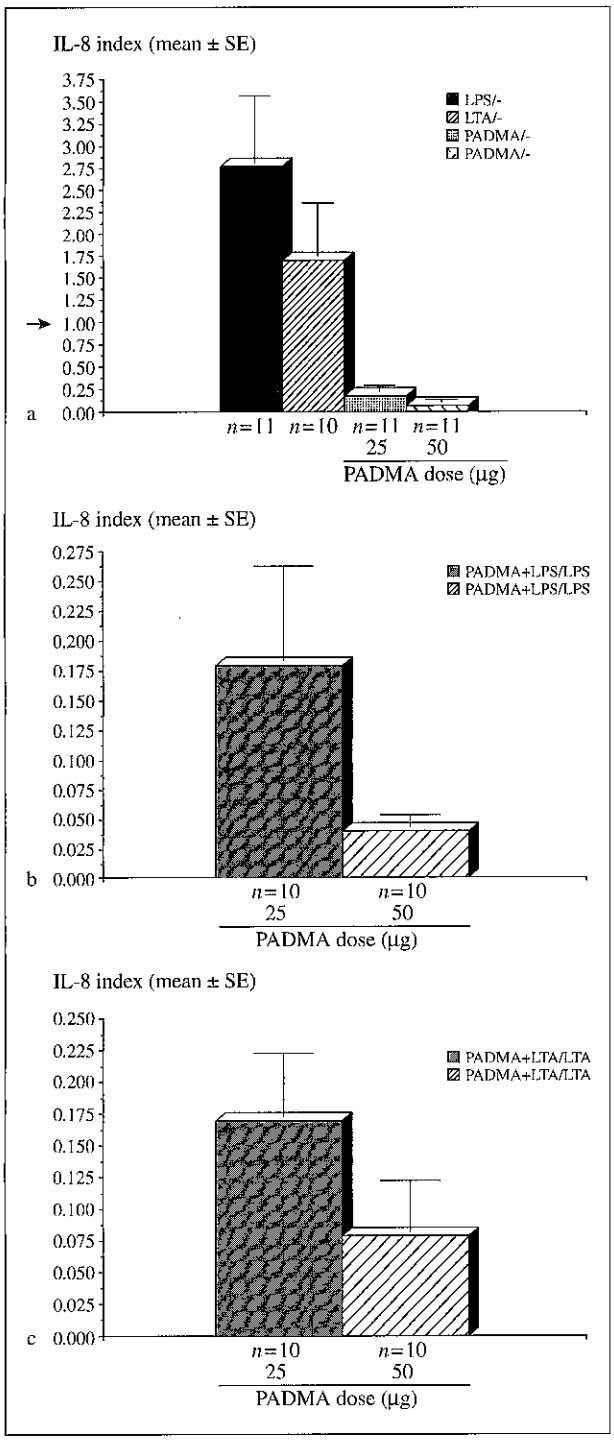

Figure 3
IL-8 production (pg/mL) by monocytes stimulated by a combination of LPS (**b**) or LTA (**c**) with PADMA-28 (25 and 50 µg) or stimulation by the individual LPS, LTA and PADMA (**a**).

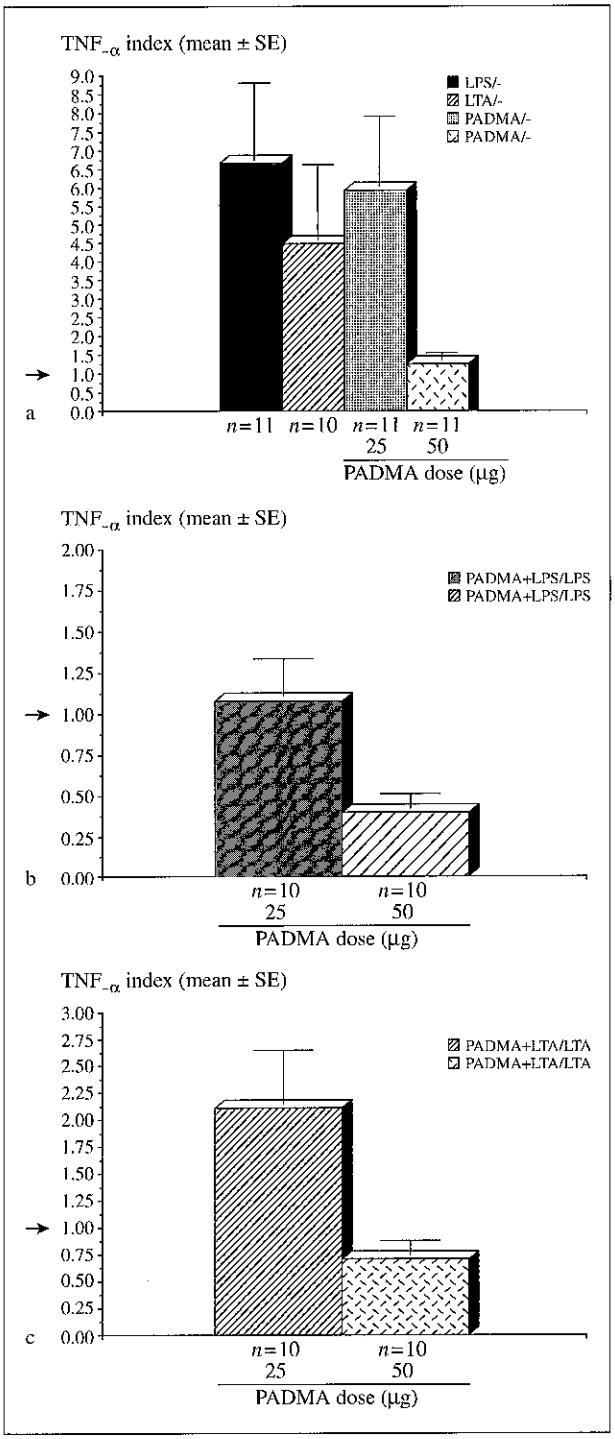

Figure 4
TNF-α production (pg/mL) by monocytes stimulated by a combination of LPS (**b**) or LTA (**c**) with PADMA-28 (25 and 50 µg) or stimulation by the individual LPS, LTA and PADMA (**a**).

phagocytes via membrane-associated TLR 2 and TLR 4 receptors [29], respectively, triggering membrane perturbation and signal transduction resulting in the secretion of a variety of proinflammatory agents including cytokines. Therefore, it is highly likely that the rich polyanionic agents in PADMA-28, (polyphenols, bioflavonoids, catechins etc.) might either bind to and neutralize LTA and LPS or block the receptors on the monocytes. Further studies to examine these possibilities are warranted.

The beneficial *in vivo* effects of PADMA-28 might also be attributed to its combined anti-oxidant [3, 7] and anti-inflammatory properties, resulting from its ability to suppress the production and activity of proinflammatory cytokines - IL-1β, IL-6, IL-8 and TNF-α [9, 15, 30] as shown in this study. However, the ability of PADMA - 28 to also lower the production of IL-10, is understandable, as the activation of monocytes affects all cytokine production by them. These effects might be of importance in the Th1/Th2 context. IL-10 characterizes Th2 responses and can suppress Th1 responses, e.g, IFN-γ and IL-2 production, thus accounting for a control mechanism, which might be affected by PADMA-28, as we have indeed shown. It is also