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A RATIONALE FOR THE PROPHYLACTIC USE OF MONOPHOSPHORYL LIPID A IN SEPSIS AND SEPTIC SHOCK

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Summary: Monophosphoryl lipid A (MLA), a substructure of bacterial lipopolysaccharide (LPS), is being developed as a prophylactic for sepsis and septic shock. In the present study it was shown that MLA induced a rapid accumulation of IFN-γ in mice that correlated with an *in vivo* priming of macrophages. Primed macrophages could be induced *in vitro* to synthesize nitric oxide, a key mediator of macrophage cytotoxicity. Due to its rapid clearance, MLA was not present in circulation at the time when IFN-γ accumulated, suggesting that MLA could not synergize with IFN-γ to systemically activate macrophages *in vivo*. MLA treatment tolerized mice against the IFN-γ response - ie., treatment of mice with MLA on day 1 blocked LPS from inducing IFN-γ on days 2-4. The significance of these results in relation to MLA's ability to enhance non-specific resistance and block LPS lethality in animals is discussed.

No effective prophylactic for human septic shock resulting from Gram-negative bacteremia is established. However, several strategies are currently being investigated in animal models (1). These include: 1) treatments with immunostimulants that enhance non-specific resistance mechanisms and limit the incidence of sepsis; 2) treatments with factors that limit the toxic effects of shock mediators; and 3) treatments with antibodies that neutralize the endotoxic properties of LPS and enhance its clearance from circulation. Generally, each treating agent is meant to impact on only one of these defense strategies. An exception is the treatment of animals with the LPS substructure, MLA. In this case, the goal is to provide two lines of defense - first, to limit sepsis by potentiating non-specific resistance; and second, to limit LPS toxicity (if sepsis occurs) by inducing tolerance to LPS. The biological feasibility for this dual defense strategy has been demonstrated in mice by showing that treatment with

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MLA protects animals against either a lethal challenge of bacterial cells or a lethal challenge of LPS (2-3). The biochemical basis for this dual defense, however, has not yet been clearly established.

Since many of the mediators of non-specific resistance also appear to mediate LPS lethality, the ability of MLA both to enhance resistance mechanisms and block endotoxic shock seems somewhat paradoxical. This paradox is perhaps most keenly represented by the contrasting activities of IFN-γ. Several models of non-specific resistance have indicated that IFN-γ is the principle cytokine required for non-specific resistance to microbial pathogens, including bacteria (4-9). On the other hand, Heinzel (10) has shown that exogenous IFN-γ, given prior to an LPS challenge, increases mortality; and that anti-IFN-γ antibody protects mice against LPS lethality. In the present study the paradox surrounding the opposing effects of IFN-γ as a mediator of both resistance and shock was addressed by comparing MLA with toxic LPS with regard to their immunostimulating and pharmacokinetic properties.

MATERIALS AND METHODS

Chemicals. Chromogenic Limulus Amebocyte Lysate kits (QCL-1000) were purchased from Whittaker Bioproducts; and mouse IFN-γ ELISA kits from Genzyme Corp. MLA (MPL immunostimulant) and *Salmonella abortus equi* LPS were from Ribi Immunochem Research, Inc. Stock solutions of MLA and LPS (1-2 mg/ml) were prepared in aqueous 0.5 % triethylamine. The mixtures were treated in a bath sonifier for 10-15 min. to enhance dispersion.

Animals. Female ICR mice aged 4-8 weeks were used for all experiments. Stock solutions of MLA and LPS were diluted with D5W immediately before injection and administered via the tail vein (0.2 ml volume).

Peritoneal Macrophage Cell Culture. Peritoneal exudates were collected in cold, RPMI 1640. Cells were pelleted by centrifugation and resuspended in culture media at a density of 1 x 10^6 cells/ml and plated on 96 well microplates (0.2 ml/well). The culture media was RPMI 1640 supplemented with fetal bovine serum (10%), amphotericin B (250 μg/l) and gentamycin (50 mg/l). After incubation for 2 hours at 37°C, nonadherent cells were removed. Adherent cells were then incubated in culture media supplemented with varying amounts of LPS or MLA for 20-24 hours.

LAL Assay. Heparinized blood was collected from mice via the ophthalmic venous plexus. The plasma was prepared according to Sturk et al. (11) with modifications. Briefly, the heparinized blood was spun at 1000 rpm for 10 minutes and the platelet rich plasma was collected. The plasma was diluted 1/10 with sterile nonpyrogenic distilled water. Diluted plasma was then heated at 75°C for 30 minutes followed by cooling to room temperature for 30 minutes. These heated, diluted plasma samples were then assayed for MLA or LPS using the LAL Chromogenic assay kit as per the manufacturer's instructions.

IFN-γ Assay. Heparinized blood was collected as above. The plasma was obtained after spinning the blood at 2000 rpms for 10 minutes. Plasma samples were then assayed using the IFN-γ ELISA kit as per instructions of the manufacturer.

Nitrite Assay. Nitrite produced by peritoneal macrophages *in vitro* was measured by the Greiss reaction (12).

RESULTS

MLA induces IFN-γ accumulation. The results in Fig. 1 confirmed earlier studies (13-14) demonstrating that LPS can induce a rapid accumulation of IFN-γ and showed that this immunostimulating property was preserved in MLA. The kinetics of induction were similar with both stimuli. Accumulation of plasma IFN-γ was initiated 4 h post-treatment, and maximum IFN-γ levels were achieved at about 6 to 8 h. The minimum dose of MLA required for IFN-γ induction was about 5 μg, while the minimum dose of LPS required was less than 1/10th this amount (data not shown). With both stimuli the accumulation of IFN-γ in plasma was transient, and none was detected in plasma samples collected 24 h post-treatment.

MLA mediates *in vivo* priming of macrophages for *in vitro* activation of nitric oxide synthesis. Synthesis of nitric oxide (NO) is a marker of

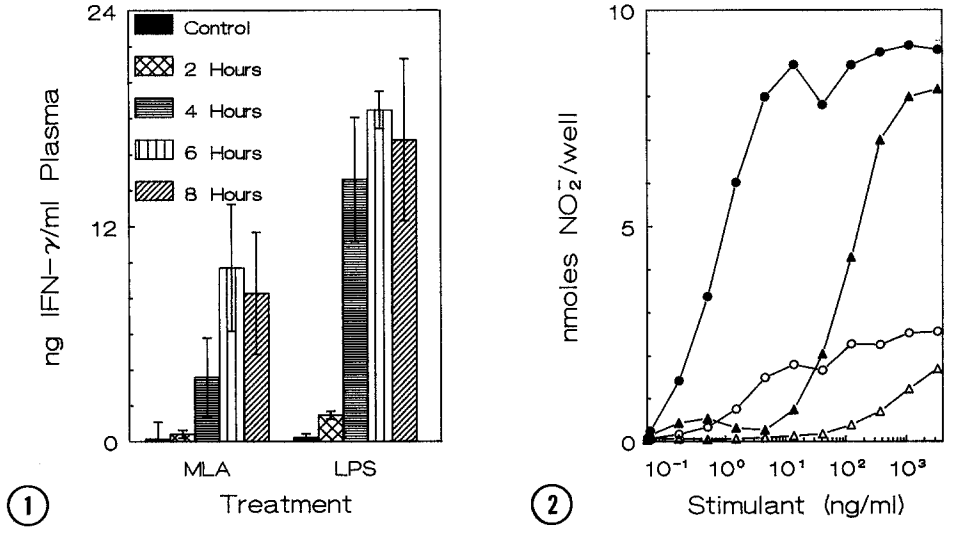

Figure 1. IFN-γ accumulation in plasma with i.v. MLA (50 μg/mouse) or i.v. LPS (10 μg/mouse). Each bar represents the mean of 5 individual responses. Standard deviations are represented by the error bars.

Figure 2. Nitrite produced by cultured macrophages. Solid symbols represent mice treated with 50 μg MLA 20 hours before cells harvested; open symbols represent cells harvested from untreated mice. Cells were stimulated either with LPS (circles) or MLA (triangles).

macrophage activation, and NO is an essential mediator of macrophage cytotoxic activity (7-8,15-16). Macrophage activation is a two step process. First macrophages must be primed by IFN-γ, and next, the primed cells must be stimulated by a second signal such as LPS. As an indirect way of assessing the biological activity of IFN-γ that was induced by MLA, we examined the state of macrophage priming in mice that had been treated with MLA. Adherent peritoneal macrophages from i.v. MLA treated mice and from untreated mice (control) were cultured in the presence of varying concentrations of either LPS or MLA, and the induction of nitric oxide synthesis was evaluated by quantifying the accumulation of nitrite in the culture media. Fig. 2 shows that macrophages from treated animals produced about 4-fold more nitrite in response to *in vitro* stimulation with either LPS or MLA than macrophages from control animals. A 100-fold higher concentration of MLA was needed to achieve half-maximum response as compared to LPS.

The rapid clearance of MLA from circulation precludes it from activating macrophages for nitric oxide synthesis *in vivo*.
LPS is known to persist in circulation for many hours after i.v. administration (16-18). In light of the synergy between IFN-γ and LPS, their coexistence in circulation could result in both systemic priming and systemic activation of macrophages. Some of the toxic effects of LPS observed in animal models of endotoxic shock could be attributable to such a synergism.

To examine whether a similar potential existed for *in vivo* synergy between MLA and IFN-γ, the kinetics of clearance of MLA was evaluated. Using a chromogenic LAL assay, the level of MLA was assessed in blood plasma at various times after i.v. injection. Fig. 3 shows that MLA was cleared very rapidly. Its apparent half-life in blood was less than 30 seconds, and an insignificant amount remained in circulation 4 h post-treatment, when IFN-γ began to accumulate in the plasma. In contrast, when 2 μg pf LPS was administered i.v., the plasma LPS concentration 4 h post-injection was about 150 ng/ml.

Treatment of mice with MLA blocks the ability of LPS to induce IFN-γ. Vogel and Henricson (19) showed that MLA induced endotoxin tolerance in mice that was manifested in the reduced potential of animals to produce cytokines in response to an LPS challenge. Cytokines blocked included CSF, TNF, IL6, and anti-viral IFNs. The results presented in Fig. 4 show that MLA also blocked IFN-γ induction by LPS. Accumulation of IFN-

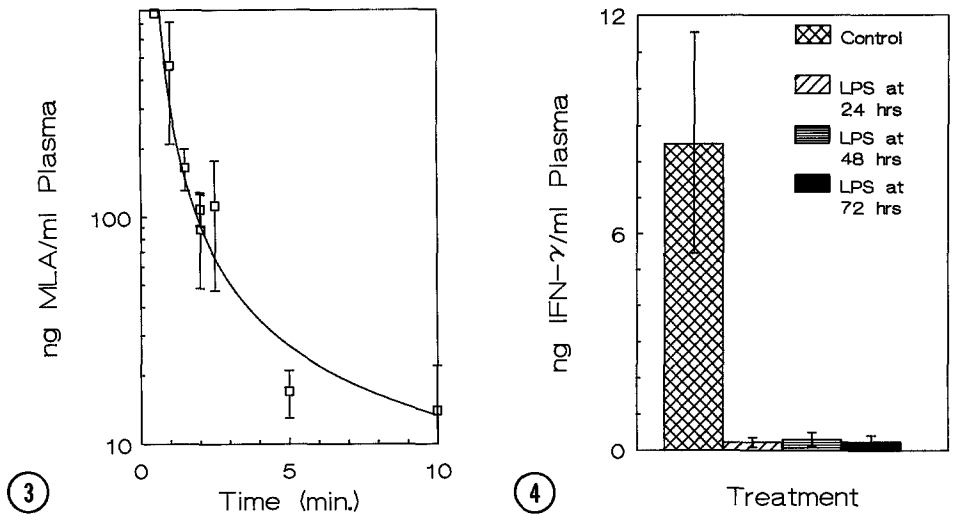

Figure 3. Clearance of MLA from mouse plasma following i.v. 20 μg MLA. Each point represents the mean of 3 determinations. Standard deviations are represented by error bars.

Figure 4. Tolerization of IFN-γ response. Mice were treated i.v. with 50 μg MLA and challenged i.v. with 8 μg LPS at designated times post-MLA. Control mice received only 8 μg LPS. Each bar represents a mean of 5 individual determinations. Standard deviations are represented by the error bars.

γ was not induced by i.v. LPS if mice had received MLA4-72 h prior to the LPS challenge.

DISCUSSION

IFN-γ induction. Vogel and Henricson (19) showed that MLA induced anti-viral IFNs, and Kiener et al. (13) showed that IFN-γ was a specific IFN induced by this LPS substructure. The present study confirms these results. Natural Killer (NK) cells, rather than T cells, appear to be the source of IFN-γ that accumulates in mice treated with LPS (14), as well as IFN-γ that accumulates in the early phase of infection of mice with *Listeria monocytogenes* (9,21). These cells are also a likely source of IFN-γ that accumulated in response to MLA. Some NK cells appear to be constitutively primed for IFN-γ production (20).

The level of IFN-γ accumulated in plasma in response to i.v. MLA was about half that obtained with i.v. LPS. Earlier work (19) showed that MLA and LPS also induced comparable amounts of CSF, but that MLA was much

less effective than LPS in inducing TNF, IL6, and anti-viral IFNs, indicating that macrophages respond differently to these signals.

Macrophage priming and NO synthesis. In non-specific responses to infection, macrophage activation appears to occur in two steps - first, IFN-γ primes macrophages; and second, other stimuli (eg., bacteria or bacterial cell wall components) activate the primed macrophages. IFN-γ appears to be an essential cofactor for the priming of macrophages to synthesize NO (7-8,15-16), and NO production is in turn essential for macrophage cytotoxic activity. The results in Fig 2. supported the view that IFN-γ, produced in response to i.v. MLA, primed peritoneal macrophages for subsequent *in vitro* activation of NO synthesis.

In the case of localized infection, both priming and activation of macrophages may be restricted to a local macrophage population. However, during sepsis both steps could occur systemically. The potential for systemic activation may be particularly acute when LPS enters the blood, because it can both induce systemic accumulation of IFN-γ and persist in circulation for sufficient time to systemically activate macrophages that were primed by this initial signal. Indeed, this unique combination of LPS traits - to rapidly induce IFN-γ and to persist in circulation so as to systemically synergize with this cytokine - may explain why endotoxic shock is the most prevalent form of septic shock in humans (1).

Rationale for MLA's biological effects. The combined properties of MLA to: a) stimulate an IFN-γ-mediated, systemic priming of macrophages (Figs. 1 and 2); b) to be rapidly cleared from circulation (Fig. 3); and c) to block subsequent induction of IFN-γ by LPS (Fig. 4) provide a rationale for its use as a prophylactic for sepsis and septic shock. Systemic priming enhances the potential of macrophages at any localized site to respond to a local infection and be activated to kill the infectious agent. Rapid clearance of MLA, together with the attenuated activity of MLA to induce proinflammatory cytokines, limits systemic macrophage activation that can cause systemic toxicity. If sepsis occurs after MLA treatment, MLA mediated tolerization of the IFN-γ response could limit adverse systemic responses to circulating LPS. According to this rationale, MLA should have an enhanced therapeutic window compared to LPS because i.v. MLA can provide a single, priming signal to macrophages, while i.v. LPS provides two overlapping signals to macrophages that both prime and activate them.

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