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In Vivo Protective Effect of Lipopolysaccharide against *Pseudomonas aeruginosa* Exotoxin A in Mice

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Lipopolysaccharide (LPS) treatment of mice 1 to 5 days prior to administration of *Pseudomonas aeruginosa* exotoxin A (PA) induced full or partial protection against PA intoxication. The optimal LPS dose that induced resistance was 50 to 100 μg per mouse. Simultaneous administration of LPS and PA to mice, however, increased their sensitivity to PA two- to fourfold. Mice pretreated with LPS demonstrated a markedly enhanced clearance rate of ^{125}I-labeled PA from peripheral blood, livers, and kidneys. In mice exposed to LPS and PA simultaneously, the rate of elimination of labeled PA was lower than that in control mice. While protein synthesis was inhibited significantly in livers and other organs of PA-exposed mice, in LPS-pretreated mice, PA-induced inhibition of protein synthesis was either diminished or totally prevented and elongation factor 2 (EF_2) levels were normal. In mice treated only with LPS, enhanced protein synthesis and increased levels of EF_2 were observed, suggesting that LPS protection against PA intoxication was perhaps a consequence of excessive amounts of EF_2 induced by LPS.

Studies carried out over more than five decades firmly established that gram-negative bacteria or components of their cell walls, such as lipopolysaccharide (LPS), cause a nonspecific increase in resistance to infection (19, 22, 25) and, in addition, tumoricidal activities (14). LPS elicits a number of biological perturbations which can lead to lethal shock. When several exotoxins from gram-positive bacteria (toxic shock syndrome toxin 1, streptococcal pyrogenic exotoxins, and staphylococcal exotoxin C) were administered with LPS, enhanced lethality of the toxins for experimental animals was observed (2, 6, 20, 24). In opportunistic infections caused by *Pseudomonas aeruginosa*, both LPS and secreted *P. aeruginosa* exotoxin A (PA) play important roles in the pathogenicities of the infections (13, 23). It is therefore of interest to study the involvement of LPS in the modification of PA toxicity in mice. In our studies, we show that concomitant exposure of mice to LPS and PA enhances the lethality of both substances. However, pretreatment of mice with LPS induces resistance to PA. The possible mechanism underlying the sensitization and increased tolerance to PA is studied.

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MATERIALS AND METHODS

Toxins. PA was purchased from the Swiss Serum and Vaccine Institute, Berne, Switzerland. The purity of the commercial toxin was analyzed on a sodium dodecyl sulfate (SDS)-polyacrylamide gel and, unless the toxin was homogeneous, it was rechromatographed on a hydroxyapatite column (9), yielding a product migrating as a single band in electrophoresis. One mouse 50% lethal dose (LD_{50}) was equivalent to 0.15 μg of PA. The LPS used in these experiments was from *Escherichia coli*. It was purchased from Difco Laboratories, Detroit, Mich., and purified by the method of Westphal and Jann (27). One LD_{50} of the LPS ranged from 500 to 600 μg per mouse, depending on the batch used.

LPS-induced-protection experiments. Charles River outbred female mice weighing approximately 25 g were treated with 100 μg of LPS, unless otherwise indicated. The LPS was administered intraperitoneally (i.p.) in 0.5 ml of saline. Control mice were injected with saline only. Control and LPS-treated mice were challenged with PA by being injected i.p. with 2.5 LD_{50}s of PA in 0.5 ml of gelatin buffer (0.2% gelatin in 0.085 M phosphate buffer, pH 6.3), and their mortality was recorded daily for 5 days.

Distribution of ^{125}I-labeled PA in control and toxin-treated mice. Iodination of PA was performed by the chloramine-T method (5), which resulted in a ^{125}I-labeled PA product with a specific activity of 5 μCi/LD_{50} of PA. The labeled PA was analyzed on a polyacrylamide gel, showing a major band with a molecular mass of 66,000 to 67,000 Da which corresponded to PA and a minor band with a molecular mass of 62,000 to 63,000 Da, most probably a breakdown product. No significant reduction in the biological activity of ^{125}I-labeled PA was observed, as shown by toxicity tests in mice.

The distribution of PA in organs of mice after various treatments was studied by injecting groups of mice with 2 LD_{50}s of nonlabeled PA plus 0.2 LD_{50} of ^{125}I-labeled PA. At various intervals following PA administration, blood was taken from the retro-orbital venous plexus into tubes containing 5 U of heparin. The mice were sacrificed by cervical dislocation, and the relevant organs were excised, rinsed with saline, blotted, and put into vials. Radioactivity in whole organs and measured aliquots of blood was counted with a Beckman gamma counter.

In vivo protein biosynthesis. Groups of control and LPS- and/or PA-treated mice were injected i.p. with either a mixture of ^{14}C-labeled amino acids (10 μCi per mouse) or [^{35}S]methionine (75 μCi per mouse; Amersham, Buckinghamshire, United Kingdom). After 3 h, mice were bled as described above and sera were separated. Mice were then sacrificed, and a piece (~500 mg) of each of the excised livers was rinsed and homogenized in 2 ml of ice-cold sucrose buffer (250 mM sucrose, 50 mM KCl, 20 mM Tris [pH 7.4], 2 mM $MgCl_2$). S10 supernatants were prepared by

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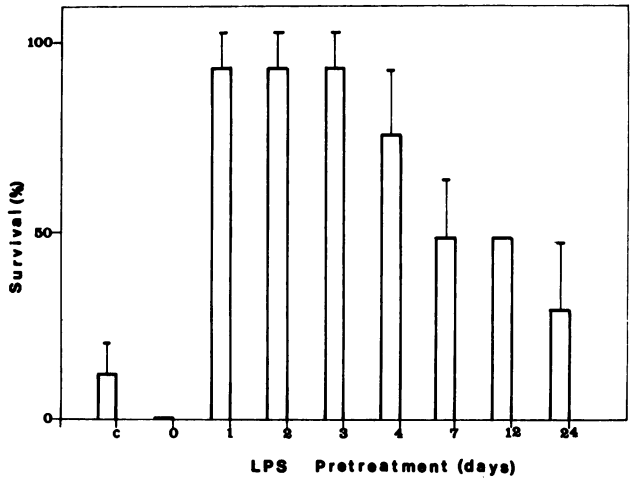

FIG. 1. Time course of LPS treatment of mice for maximal protection against PA intoxication. Groups of six mice were injected with 100 μg of LPS per mouse prior to PA injection. At day 0, all groups, including a non-LPS-treated control group, were injected i.p. with 2.5 LD_{50}s of PA per mouse. Data are the means of four experiments.

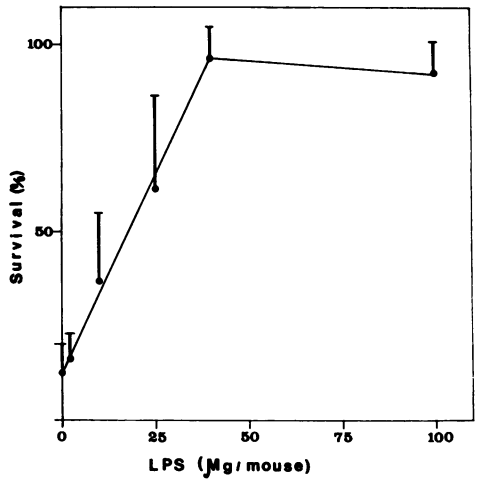

FIG. 2. LPS dose effect on resistance of mice against PA. Groups of six mice were injected i.p. with various amounts of LPS 2 days prior to PA challenge (3 LD_{50}s per mouse). The data presented are the means of three experiments. Treatment with 25 μg of LPS per mouse already indicates a significant protection against PA ($P < 0.01$).

centrifugation of homogenates at 10,000 rpm for 15 min. Amino acid incorporation was determined on duplicate 50-μl aliquots of sera or S10 supernatants that were absorbed on filter disks and trichloroacetic acid (TCA) precipitated as described elsewhere (28). Results were expressed as the mean radioactivity incorporated per milliliter or gram of tissue in a mouse group.

Extraction and quantitation of mouse liver EF_2. Livers from groups of four mice were homogenized separately, essentially as described by Gill and Dinius (7). Endogenous NAD was removed from the supernatants, and the samples were assayed for active elongation factor 2 (EF_2). The ADP ribosylation transferase in these assays was PA activated by the method of Leppla (9), and the assays were carried out essentially by the method of Pavlovskis et al. (16). Reaction mixtures in a final volume of 50 μl contained the following: 15 μl of buffer (0.05 M Tris buffer [pH 8.4]–0.04 M dithiothreitol), 1 μg (10 μl) of activated PA, 25 μl of liver extract containing the tested EF_2, and 0.05 μCi (in 5 μl) of [U-^{14}C]NAD (specific activity, 299 mCi/mmol; Amersham). After incubation for 15 min at 37°C, 40-μl aliquots of each incubation mixture were transferred to filter paper disks and processed as described for amino acid incorporation, except for the heating step. Preliminary PA dose dependence experiments were carried out to ensure the presence of saturated quantities of activated PA in reaction mixture assays. All assays were done in duplicate. The average active EF_2 content in livers from various mouse groups was calculated as described by Gill and Dinius (7).

Statistical analysis. Statistical analyses were performed by the paired Student *t* test.

RESULTS

Effect of LPS pretreatment on PA-induced lethality to mice. Groups of six mice were injected with 100 μg of LPS per mouse, and at various times later, they were exposed to 2 to 3 LD_{50}s of PA (Fig. 1). This pretreatment of mice with LPS afforded protection from PA-induced lethality to mice. The degree of protection depended on the length of time of LPS pretreatment relative to that of PA administration. Survival of more than 80% of PA-challenged mice that had been exposed to LPS treatment 1 to 3 days beforehand was observed. The LPS induced a gradually decreased protective state which reached almost a control state 24 days thereafter (Fig. 1). The LPS-induced resistance to PA intoxication was dose dependent, with maximal protection of mice achieved with 40 to 100 μg of LPS (Fig. 2). Treatment of mice with 25 μg of LPS induced a partial but significant resistance to PA ($P < 0.01$). Mice pretreated with small doses of LPS (2 to 10 μg of LPS per mouse) also acquired certain levels of resistance to PA challenge; these results, however, were inconsistent. The exposure of mice to LPS and PA simultaneously caused a roughly threefold enhancement in the lethality to mice (Table 1).

Uptake and clearance of ^{125}I-labeled PA in blood and organs of LPS-treated and control mice. Changes in rates of clearance of PA by the reticuloendothelial system of LPS-treated mice may explain the observed resistance of these mice to PA. To test this possibility, the distribution of ^{125}I-labeled PA in various tissues of control and LPS-treated mice was examined as a function of time.

From the data presented in Fig. 3, it is apparent that during the first half hour after toxin administration, the transfer of the toxin from the peritoneal cavity to the peripheral bloodstream and the organs tested is highly accelerated in LPS-pretreated mice (particularly at 48 h) as

TABLE 1. LPS-induced susceptibility of mice to PA[a]

PA dose (ng/mouse)	% Mortality of mice challenged with:	
	PA	PA + LPS
350	83.3 ± 11.8	100
200	33.2 ± 12.0	83.3 ± 13.6
115	0	54.1 ± 8.3

[a] Groups of six mice were injected i.p. with PA (as indicated) and 100 μg of LPS in 0.5 ml of phosphate-buffered saline. Mortality of mice was recorded daily for 5 days. Data are the means of four experiments.

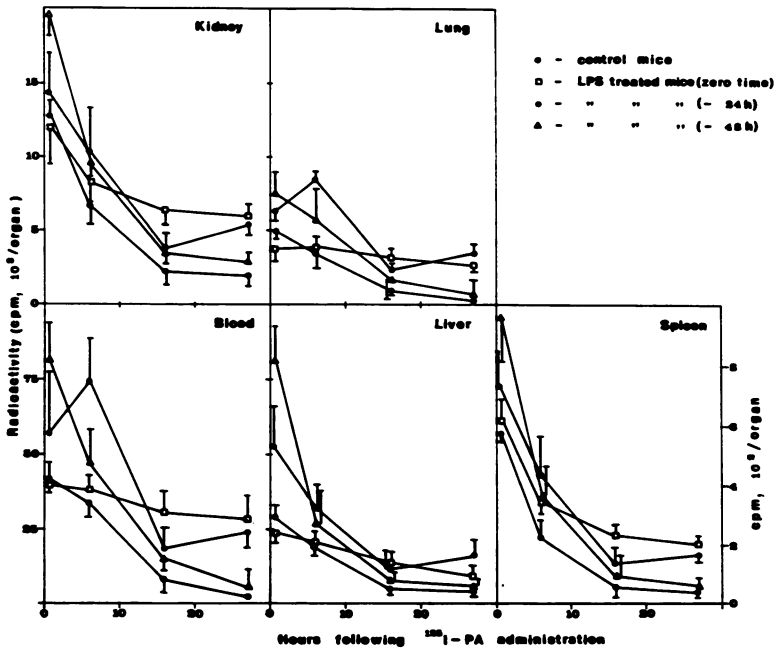

FIG. 3. Uptake and clearance of ^{125}I-labeled PA in blood and organs of control and LPS-treated mice. Groups of five mice were pretreated with 100 μg of LPS per mouse at the indicated times. At time zero, all mice, including a control group, were each injected i.p. with 0.2 LD_{50} of ^{125}I-PA (5 μCi/LD_{50} of toxin). At various times following PA administration, blood was taken into tubes containing 5 U of heparin. The mice were sacrificed, and the relevant organs were removed into vials. Radioactivity in whole organs and measured aliquots of blood was determined with a Beckman gamma counter. The data are the means of radioactivity in organs or blood of five mice.

opposed to mice exposed to LPS and PA simultaneously and control mice. As a result, the toxin concentration in peripheral blood and the organs analyzed in the mice treated with LPS for 48 h roughly doubled. The accumulation of the toxin was enhanced to a lesser extent in tissues of the mice pretreated with LPS for 24 h, with significant increment values observed for blood and liver specimens (blood, 50.5% ± 21.8%, $P \leq 0.01$; liver, 76.6% ± 27.8%, $P \leq 0.01$). The distribution of toxin in mice, when administered simultaneously with LPS, did not differ from that in control mice. During the next 6 h, the rate of clearance of the ^{125}I-labeled toxin (as judged by the slopes of curves in Fig. 3) from peripheral blood, livers, kidneys, and spleens was markedly enhanced in the 48-h-pretreated mice. The consequence of this accelerated clearance was that despite the higher initial accumulation of toxin in the organs, 6 h after challenge, the distribution of toxin in the mice treated with LPS for 48 h was comparable to that in control mice. The highest concentrations of toxin, 6 h after PA administration, were found in tissues of the mice treated with LPS for 24 h. This is most probably because of a continuous flow of toxin from the peritoneum to other parts of the body (see the curves for 24-h-LPS-treated mice in Fig. 3, blood and lung specimens). Further decay of the labeled toxin in the various tissues of the LPS-pretreated mice was much the same as in the control mice, reaching after 24 h ^{125}I-labeled-toxin levels of approximately 10% of the initial amounts. Only in the case of mice simultaneously exposed to LPS and PA was the rate of clearance of the ^{125}I-labeled toxin in the various tissues significantly lower. The amounts present in blood or organs after 24 h were as follows: blood, 59.6% ± 24.3%; liver, 43.8% ± 19.3%; kidney, 30.5% ± 10.7%; spleen, 18.6% ± 7.4%; and lung, 66.0% ± 31.2% (Fig. 3).

In arbitrarily chosen blood samples taken at various times (30 min to 24 h), it was shown that the label was confined to the plasma and that more than 80% of it was TCA precipitable. The concentrations of radioactive toxin obtained in the various tissues of control and LPS-treated mice were too low for further study of the integrity of the ^{125}I-labeled toxin as a function of time.

The effect of PA on protein biosynthesis in LPS-treated mice. The fact that the toxicity of PA is closely associated with the inhibition of protein biosynthesis is well established (11, 15, 17), and the liver is the main target of this inhibition (17). In order to understand the protective effect of LPS pretreatment on PA intoxication of mice, we compared levels of newly synthesized proteins in livers and sera of LPS-pretreated (48 h) and control mice. The results demonstrate a massive inhibition of newly synthesized proteins occurring 24 h after PA administration to mice (Table 2, Fig. 4); similar results have already been presented by other groups (13). This inhibition of protein biosynthesis can be prevented if mice are pretreated with LPS (Table 2, Fig. 4). LPS treatment alone induced enhancement of protein biosynthesis, which was evident as early as 24 h after LPS administration (Fig. 5). The pattern of serum proteins synthesized in mice after LPS treatment alone or in combination with PA did not differ significantly from the pattern seen for normal mice (Fig. 4, lanes a and c through f). In the presence of PA only, however, most of the proteins synthesized in serum were inhibited (Fig. 4, lane b), except for two proteins, one of which (molecular weight, 22,000) was found in normal quantities, while the synthesis of the other one (molecular weight, 44,000) was enhanced.

EF_2 levels in LPS-treated mice. A series of well-established experiments performed previously provided evidence that injection of mice with PA or, alternatively, with *P. aeruginosa* toxigenic strains producing this toxin substantially

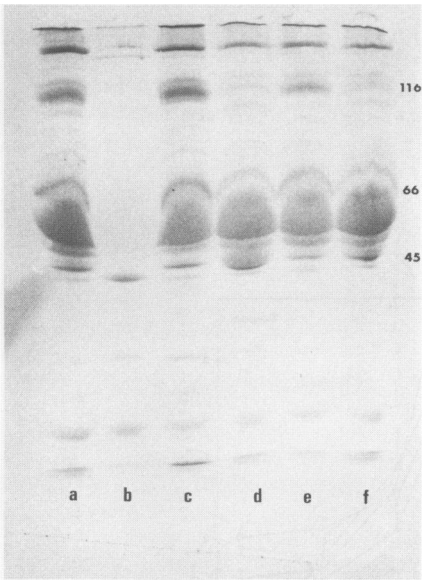

FIG. 4. Polyacrylamide gel electrophoretic analysis of [^{35}S]methionine-labeled serum proteins from PA-challenged, control, and LPS-treated mice. Groups of four mice treated either with LPS or PA alone or sequentially with both components were injected with 75 μCi of [^{35}S]methionine according to procedures described in Materials and Methods. Three hours after [^{35}S]methionine administration, the pooled sera from the various groups, containing ~30,000 cpm (15 to 70 μl), were subjected to SDS-polyacrylamide gel electrophoresis. Serum samples from the groups of mice were analyzed as follows: lane a, LPS treated (48 h) and PA challenged (24 h); lane b, PA challenged; lane c, control; lane d, LPS treated (48 h); lane e, LPS treated (24 h); lane f, LPS treated (zero time). Molecular weights (in thousands) are indicated on the right.

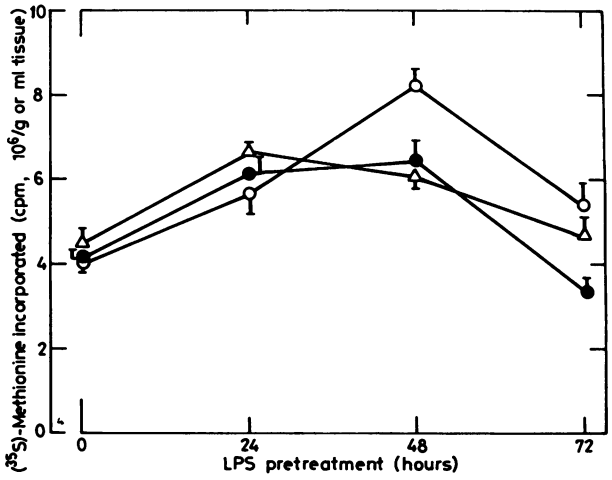

FIG. 5. Enhancement of protein biosynthesis in tissues of LPS-treated mice. Groups of four mice treated with LPS at the indicated times were injected with [^{35}S]methionine, and 3 h later, TCA-precipitable radioactivity was determined in the serum, liver, and spleen extracts. Symbols: △, serum; ○, liver; ●, spleen.

reduced EF_2 levels in the livers of these animals, resulting in mortality (16). These experiments, along with our observations that LPS-induced protection against PA intoxication paralleled the increase of the capacity of protein synthesis in treated mice (see paragraph above), encouraged us to examine the levels of active EF_2 in livers of control and LPS-treated mice. EF_2 activity in individual liver extracts of control mice, PA-challenged mice, and PA-challenged mice treated (48 h) with LPS prior to PA administration was determined. The results presented in Table 3 show unequivocally that PA challenge drastically reduced the level of active EF_2 (84.9% ± 14.2%; $P < 0.01$). The level of active EF_2 in livers of LPS-treated mice (48 h) is elevated, however, compared with levels in livers of control mice (141.9% ± 17.3%; $P < 0.05$). EF_2 in livers treated for 24 h with LPS nearly doubled (193.8% ± 21.4%; results not presented). Livers from mice pretreated with LPS and subsequently exposed to PA contained less active EF_2 than livers of control mice (42.7% ± 15.3%) but three times as much as the livers of mice treated with PA only (Table 3).

TABLE 2. Effect of LPS on inhibition of protein synthesis by PA in vivo[a]

LPS pretreatment (h)	PA administration (LD_{50})	^{14}C-amino acid incorporation in:	
		Liver (cpm/g)	Serum (cpm/ml)
		73,670 ± 23,490	93,160 ± 18,611
	2	21,080 ± 3,394	23,030 ± 5,218
48	2	82,750 ± 10,451	110,120 ± 12,558
48		113,460 ± 25,320	184,680 ± 36,402

[a] Two groups of four mice were injected i.p. with PA on day 0. Two groups were treated i.p. with 100 μg of LPS 2 days before PA exposure. One day after PA injection, all mice, including a control group that did not receive any treatment, were injected with 20 μCi of ^{14}C-amino acid mixture. Three hours later, the mice were sacrificed, and radioactivity in tissue was determined as described in Materials and Methods. Data are means ± standard deviations of amino acid incorporation per gram of the organ or per milliliter of serum of each of the four mice in each group.

DISCUSSION

Conditions for treatment invoking resistance to PA intoxication in mice were determined in the present study. Maximal protection against PA was achieved by treating mice with a dose of 50 to 100 μg of LPS 48 h prior to PA challenge. The LPS-induced protective state gradually declined, reaching a normal state 3 weeks thereafter. These findings are reminiscent of the well-established observations that gram-negative bacteria or components of their cell walls induced a short-lived nonspecific resistance to infections (reviewed in references 11, 15, and 17). Our observation that exposure of mice to LPS prior to PA administration renders them resistant to a lethal dose of this toxin illuminates another facet of

TABLE 3. Effect of LPS treatment of mice on the level of functional EF_2 in liver after PA challenge[a]

Group no.	LPS pretreatment (h)	PA exposure (LD_{50}/mouse)	EF_2 in liver	
			nmol/g of liver	% (relative to control)
1			1.13 ± 0.06	
4	48		1.61 ± 0.04	141.9
5	24		2.19 ± 0.07	193.8
2		2.4	0.13 ± 0.06	11.5
3	48	2.4	0.48 ± 0.19	42.7

[a] All five groups of mice were sacrificed 24 h after PA administration, and 2 to 3 g of their liver tissues was used for preparation of extracts, in which EF_2 content was determined. Data are the means of EF_2 levels in five extracts prepared separately from five livers from a specific group.

a complex phenomenon underlying the induction of nonspecific resistance to gram-negative bacteria, in which exotoxins are considered to play an important role in their pathogenicity (4, 10).

Simultaneous administration of LPS and PA to mice increased their susceptibility to PA (Table 1). It is pertinent that such synergistic effects between LPS and exotoxins in enhancing lethality have also been recorded for other exotoxins (2, 6, 20, 24). Taking into consideration the multipotent activity of the LPS molecule (reviewed in reference 12), it is not surprising that changing the schedule of its administration may induce either enhanced lethality or resistance to PA.

The observed synergism between LPS and some exotoxins, including in our case PA, in enhancing lethality was attributed mainly to a block in the clearance functions of the reticuloendothelial system (2, 6, 24). Our experiments, in which we monitored the distribution of ^{125}I-labeled PA administered with LPS, essentially supported this hypothesis, namely, that the elimination of labeled PA from the various examined organs of mice treated with LPS and PA simultaneously was significantly slower than in control mice (Fig. 3).

Contrary to the case of simultaneous LPS and PA administration, LPS treatment prior to PA exposure enhanced the clearance of the labeled PA. The well-established fact that LPS activates the reticuloendothelial system (including macrophages and Kupffer cells) through a whole series of induced mediators (1, 3, 18) can well explain the enhanced removal of the exotoxin from the various tissues examined. This alone, however, could not explain the increased resistance to PA after LPS treatment, because the amounts of toxin found in the organs of control and LPS-pretreated mice did not differ significantly at any time (Fig. 3). Such an explanation could still hold if the TCA-precipitable toxin, found in the LPS-treated mice, is partially degraded and therefore biologically inactive. Further studies employing more sensitive detection of the toxin will be necessary to verify this assumption.

Another explanation for the mechanism of LPS-induced resistance to PA could be polyclonal activation of existing clones producing natural antibodies, including those to PA. This possibility was excluded because no detectable antibodies (neither immunoglobulin G nor immunoglobulin M) directed to PA were observed by a solid-phase immunoassay in sera of mice taken between 1 and 4 days post-LPS treatment (results not shown). Our results were in agreement with observations described by other investigators demonstrating that LPS pretreatment suppressed the response of antibody production (1, 21).

Since the toxic action of PA is attributed mainly to its inhibitory effect on protein biosynthesis, the liver being the main target (15), we speculated that the LPS-increased resistance to PA activity is correlated primarily with changes in protein biosynthesis triggered by LPS. Our results clearly indicated that in mice treated with LPS, the output of newly synthesized proteins more than doubled in livers and increased to a lesser extent in sera and spleens (Fig. 5); similar results were also described by other investigators, particularly for acute-phase proteins (3, 8, 26). Moreover, we have shown that, in addition, LPS increases the activity of EF_2, probably concomitant with the expansion of the whole protein-synthesizing machinery. In order to annihilate protein synthesis in tissues with excess amounts of EF_2, the tissues should be exposed to increased quantities of PA, which was the case in our experimental model. In PA-intoxicated mice, no de novo-synthesized proteins were detected in livers or sera, whereas in LPS-pretreated mice, PA intoxication caused only partial protein synthesis inhibition, probably enabling the mice to survive. The state of protein synthesis in the different schedules of mouse treatment was a reflection of EF_2 levels found in the various tissues examined (Tables 2 and 3). Major differences in the pattern of the newly made proteins in LPS-treated mice were not found by one-dimensional gel electrophoresis analysis. It is known, however, that changes in serum proteins occur during sepsis (8, 26), suggesting that further studies by improved methods might reveal altered protein patterns perhaps involved in the induction of increased tolerance to PA. In the case of challenge with only PA, protein synthesis is stopped almost completely, as is expected (11, 13, 15–17), except in the case of continuous synthesis of two proteins (Fig. 4, lane b) which may perhaps be involved in the pathogenesis of PA toxemia.

In conclusion, it is suggested that decreased susceptibility of LPS-treated mice to PA is a consequence of an activated reticuloendothelial system responsible for the enhanced clearance and perhaps degradation of the toxin. An additional explanation for the increased tolerance to PA in LPS-treated mice is the induced enhancement of protein synthesis concomitant with the elevation of EF_2 activity that counterbalances the inhibitory effect of PA in this process.

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