ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1994, p. 2671–2675
0066-4804/94/$04.00+0

Vol. 38, No. 11

Unilamellar Liposomes Modulate Secretion of Tumor Necrosis Factor by Lipopolysaccharide-Stimulated Macrophages

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Received 5 April 1994/Returned for modification 1 July 1994/Accepted 11 August 1994

Liposomal encapsulation of antimicrobial agents has been used to improve drug delivery, particularly against intracellular pathogens. The effect of unilamellar liposomes on macrophage activation in response to *Escherichia coli* lipopolysaccharide was examined. Liposomes caused a dose- and time-dependent inhibition of tumor necrosis factor release by lipopolysaccharide-treated cells. The accumulation of tumor necrosis factor mRNA transcripts was unaffected, suggesting a posttranscriptional mechanism for this effect. However, induction of macrophage procoagulant activity was unaffected by liposomes, indicating a selective rather than a global inhibition. These data suggest that liposomes used for drug delivery may modulate the host response to infection.

Standard therapy for the management of secondary bacterial peritonitis includes fluid resuscitation to restore normal hemodynamics, surgical intervention to deal with the underlying pathological process and to reduce the bacterial inoculum, and the use of antimicrobial agents to treat local and systemic infections. Despite modern therapeutic approaches, clinical failures occur, some of which are attributed to antibiotic failure. The reasons for the lack of efficacy of various antimicrobial regimens include bacterial resistance, inadequate antibiotic levels, the failure of antibiotics to penetrate into sites of residual infection, and the reduced levels of antibiotic activity within the abnormal microenvironment of infection. Various novel approaches have been used to overcome some of these deficiencies. Liposome-encapsulated antibiotics have been shown to have improved penetration into phagocytic cells and have been advocated for use against intracellular microbes (1, 22). Indeed, this carrier system augmented the efficacy of various antimicrobial agents against a wide range of microorganisms that survive within macrophages (1, 6, 9, 25). Our laboratory has previously reported that the use of cefoxitin encapsulated within unilamellar liposomes (95% dipalmitoylphosphatidylcholine, 5% phosphatidylserine) significantly reduced the mortality rate compared with that with the use of free cefoxitin in an intra-abdominal infection model in the rat (5). Improved survival correlated with a reduction in residual bacterial numbers in the peritoneal cavity and the liver, suggesting that the beneficial effect of liposome-encapsulated cefoxitin was mediated via the improved microbicidal activity.

Experimental intra-abdominal infection has been shown to initiate the release of numerous cytokines by cells of the monocyte/macrophage lineage (11). Among these, tumor necrosis factor (TNF) appears to play a central role in the host response to infection, including the lethality associated with endotoxemia (3, 13, 17, 26, 27). TNF infusion causes death in experimental animals and precisely mimics the pathophysiological changes produced by endotoxemia. In addition, treatment with anti-TNF antibodies prevents the hemodynamic and pathological alterations as well as the lethal effects of endotoxin administration. On the basis of the importance of TNF in the development of the host response to intra-abdominal infection, we hypothesized that the salutory effect of liposome administration might in part be accounted for by virtue of its ability to blunt the release of TNF by macrophages. The present studies demonstrate that liposomes cause a dose-dependent reduction in lipopolysaccharide (LPS)-induced TNF secretion. This effect appeared to be due to a posttranscriptional event rather than an effect on the signaling mechanisms leading to LPS-induced TNF gene transcription.

Specific-pathogen-free female Swiss Webster mice (ages, 6 to 8 weeks) were obtained from Taconic Farms. Peritoneal macrophages were harvested 4 to 5 days after intraperitoneal injection of 2 ml of thioglycolate. Cells were recovered by peritoneal lavage and were washed twice with Hanks balanced salt solution without calcium. Following enumeration with a Coulter Counter model Z_F, cells were resuspended in RPMI 1640 with 10% fetal calf serum at a final concentration of 10^6 cells per ml. The cell population was consistently >85% macrophages by Wright's staining, and viability was consistently >95% by trypan blue exclusion at the end of the incubation period. Thioglycolate-elicited macrophages were used to model the state of activation of cells which might be present in the peritoneal cavity during peritoneal inflammation. Cells were stimulated with *Escherichia coli* LPS (1 μg/ml) in the presence or absence of liposomes at 37°C in 5% CO_2. At the indicated time points, cells were pelletted and the supernatant was aspirated and frozen at −70°C for later determination of TNF. Cells were then resuspended in RPMI 1640 and were frozen for determination of procoagulant activity (PCA). In some studies, RNA was extracted from cell pellets for subsequent Northern (RNA) blot analysis. Liposomes were prepared by using a 9.5:0.5 molar ratio of dipalmitoylphosphatidylcholine and phosphatidylserine. The lipids were mixed in chloroform-methanol (2:1) and were dried in vacuo. The dried lipid film was rehydrated in warmed sterile saline to 124 mM, and the resulting vesicles were extruded 10 times through

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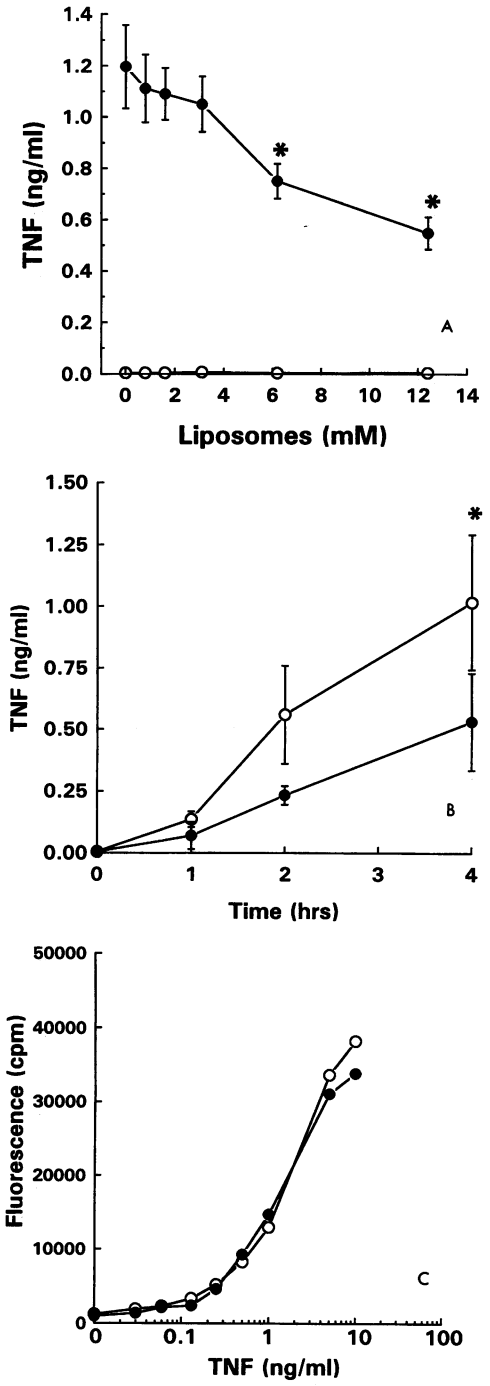

FIG. 1. Effects of liposomes on TNF release by macrophages. (A) Macrophages were incubated with various concentrations of liposomes in the presence or absence of LPS (1 μg/ml) for 4 h. The cells were pelleted, and the supernatant was used for measurement of the TNF concentration by enzyme-linked immunoadsorbent assay. The data represent the means ± standard errors of nine experiments, each of which was performed in duplicate. Symbols: ○, no LPS; ●, plus LPS; *, $P < 0.05$ versus no liposomes by analysis of variance. (B) Macrophages were treated with LPS (1 μg/ml) in the presence or absence of liposomes (6.2 mM). At times of 1, 2, and 4 h, the cells were pelletted and the supernatant was used for the measurement of TNF by enzyme-linked immunoadsorbent assay. The data represent the means ± standard errors of four experiments, each of which was performed in duplicate. Symbols: ○, LPS alone; ●, LPS plus liposomes; *, $P < 0.05$ versus no liposomes at the same time point by analysis of variance. (C) TNF standard curves in the presence (○) or absence (●) of liposomes. Murine recombinant TNF (0 to 10 ng/ml) was added to wells coated with monoclonal hamster anti-murine antibody in the presence or absence of liposomes (6.2 mM), and the enzyme-linked immunoadsorbent assay for TNF was performed as described in the text. Liposomes had no effect on the assay system. Results of a representative study are shown.

doubly stacked 400-nm polycarbonate filters, yielding large unilamellar vesicles.

The TNF concentration in the supernatants was measured by enzyme-linked immunoadsorbent assay. The antibody sandwich was detected by fluorescence (10). Microtiter plates were coated with a monoclonal hamster anti-murine TNF antibody (Genzyme, Boston, Mass.), incubated with samples, washed, and then exposed to the polyclonal rabbit anti-murine TNF antibody (Genzyme). The enzyme sandwich was then incubated with goat anti-rabbit immunoglobulin G alkaline phosphatase (Jackson, West Grove, Pa.) for 1 h at room temperature prior to the addition of the substrate solution, 5-fluorosalicyl phosphate, and the developing reagent, Terbium-EDTA. The fluorescence was measured with a time-resolved fluorometer. The calibration curve with the murine TNF standard (Genzyme) and the data reduction were performed by using an automatic immunoanalyzer (Cyber Fluor 615). The assay was sensitive to a level of 30 pg/ml. PCA in freeze-thawed macrophages was determined by measuring their capacity to shorten the spontaneous clotting time of normal citrated human plasma in a one-stage clotting assay, as described previously by us and others (16, 24). Clotting times were converted to milliunits of PCA by comparison with the clotting times of a rabbit brain thromboplastin standard in which 36 mg (dry weight) per ml was assigned a value of 100,000 mU of PCA. Previous studies have shown that PCA induced with *E. coli* LPS has thromboplastin-like activities, making comparison with a thromboplastin standard valid (23). The induction of PCA from a baseline of 200 mU/10^6 macrophages to 940 mU/10^6 macrophages in cells stimulated by LPS alone represented a shortening of the clotting time from 85 to 67 s. The total cellular RNA was extracted from 10^7 cells per group by the method of Chomczynski and Sacchi (8). After electrophoresis, RNA was transferred to Immobilon, hybridized with the ^{32}P random-labeled cDNA probe for murine TNF (provided by Bruce Beutler [19]), and washed under high-stringency conditions. The blots were exposed overnight to X-OMAT film, and the film was developed. Comparable RNA loading between lanes was assured by probing with a cDNA probe for α-tubulin (provided by Dan Drucker [15]).

Liposomes caused a dose-dependent inhibition of LPS-induced TNF secretion (Fig. 1A). Figure 1B illustrates the time course of inhibition and shows that the effect of liposomes was evident in as early as 2 h. The inhibitory effect did not appear to be due to cytotoxicity (trypan blue exclusion, >90%) or to interference with the assay system. As shown in Fig. 1C, the standard curve for TNF over the concentration range of 0.1 to 10 ng/ml was not altered by the presence of liposomes (6.2 mM). To rule out the possibility that liposomes incubated with macrophages might interfere with the recovery of TNF from the aspirated supernatants, known concentrations of TNF were added to solutions containing both liposomes and macrophages. Following incubation at 37°C, the supernatant was aspirated and the TNF concentration was measured. Over the range of 0 to 1.0 ng/ml, the concentration of TNF measured in

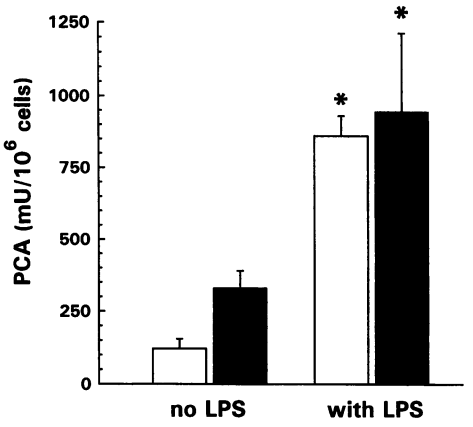

FIG. 2. Effects of liposomes on LPS-induced PCA. Macrophages were incubated with (□) or without (■) liposomes (6.2 mM) in the presence or absence of LPS (1 μg/ml) for 4 h. The cells were pelleted, frozen at −70°C, and later assayed for PCA by the one-stage recalcification assay as outlined in the text. The data represent the means ± standard errors of four experiments, each of which was performed in duplicate.

the supernatant did not differ from that added initially (data not shown).

To discern whether the inhibitory effects of the liposomes were due to the global suppression of cell function, PCA expression was evaluated (Fig. 2). In contrast to TNF, liposomes had no effect on the ability of LPS to stimulate macrophage PCA, although in the absence of LPS, a small increase in PCA was induced. Liposomes had no effect on the recalcification system used to measure PCA (data not shown).

The mechanism of liposome-mediated inhibition of TNF release was studied by evaluating their effects on the levels of TNF transcripts by Northern blot analysis. Figure 3A shows that LPS caused a marked increase in TNF mRNA. However, the addition of liposomes to LPS-treated cells had no effect on the LPS-induced rise. Data derived from four separate studies are summarized in Fig. 3B.

Liposomal encapsulation of antimicrobial agents has proven to be an effective means of drug delivery, particularly against intracellular pathogens (1, 9). Phagocytic uptake of liposomes facilitates exposure of these microorganisms to concentrations of drug which are not normally achieved by incubation of cells with the free antibiotic and results in enhanced microbial killing (25). In addition to antimicrobial agents, the host response to infection is crucial to the effective clearance of invading pathogens. TNF, released predominately by cells of the monocyte/macrophage lineage, is considered one of the pivotal cytokines in the initiation of the response (13, 26). The present studies demonstrated that liposomes inhibited LPS-induced TNF release from peritoneal macrophages. The effect was not due to cytotoxicity since the cells retained their ability to exclude trypan blue and also expressed PCA normally in response to LPS stimulation. Furthermore, liposomes did not interfere with the TNF assay or the ability of TNF to be recovered from the supernatant of LPS-stimulated cells. The cellular mechanisms underlying the effects of liposomes on TNF secretion were examined. The observation that liposomes had no effect on the LPS-induced accumulation of TNF transcripts suggests that they exerted their inhibition at a posttranscriptional level. However, the normal expression of PCA in response to LPS demonstrates that this was selective rather than global inhibition of posttranscriptional mechanisms. Similarly, studies by Bakouche and colleagues (2) demonstrated that LPS mixed with multilamellar vesicles has no effect on interleukin-1 (IL-1) production by human monocytes, although the secretion of IL-1 was mildly reduced. Since

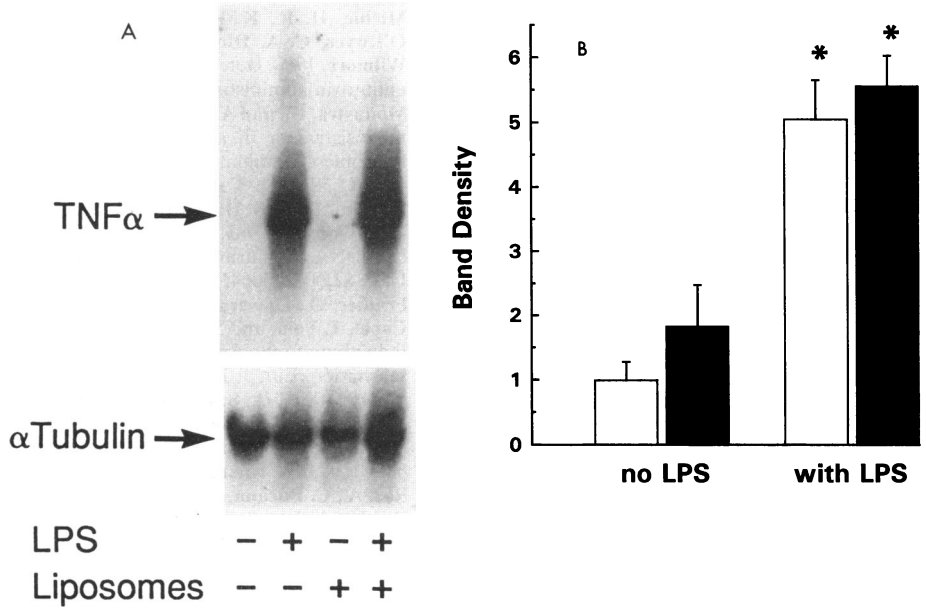

FIG. 3. Effects of liposomes on level of TNF mRNA. (A) Macrophages were incubated with or without liposomes (6.2 mM) in the presence or absence of LPS (1 μg/ml) for 4 h. The cells were pelletted, and total RNA was extracted for Northern blot analysis by using a probe for murine TNF. Equal mRNA loading was confirmed by using a probe for α-tubulin. A representative blot of four separate experiments is shown. (B) Laser densitometry of Northern blots for TNF. Data represent the means ± standard errors of four experiments. *, $P < 0.05$ versus no LPS.

PCA remains cell associated, an effect of liposomes on its secretion would not have been detected. Further studies evaluating the effects of liposomes on the posttranscriptional processing of TNF transcripts are required.

Many of the previous studies investigating the effects of lipids on LPS-mediated stimulation of the cytokine response have focused on the ability of various lipoproteins to bind LPS and thus precluded study of its interaction with monocytes/macrophages (7, 12, 29). The finding that LPS induces comparable levels of TNF transcripts and PCA activity in both the presence and the absence of liposomes demonstrates that LPS inactivation by liposomes is an unlikely contributor to the observed effect. The same observations make it unlikely that inhibition of TNF production by liposomes is due to LPS tolerance in liposome-treated cells or, alternatively, to the induction of an immunosuppressive cytokine such as IL-10. LPS tolerance with respect to TNF secretion is characterized by reduced levels of gene transcription (28), while in the present studies TNF mRNA levels were equivalent in LPS-treated cells in the presence or absence of liposomes. Furthermore, tolerance with respect to PCA occurs in parallel to TNF (14). However, liposomes had no effect on PCA. Similarly, IL-10 suppresses TNF secretion by reducing the level of mRNA transcripts (4) and also inhibits TNF and PCA in parallel (20). By contrast, another immunosuppressive cytokine, transforming growth factor β, appears to reduce TNF secretion by suppressing translation (4). However, this cytokine would have been expected to inhibit PCA in parallel with TNF (21).

The in vivo relevance of the present studies requires further investigation. The present studies provide a cellular mechanism for observations recently reported by Monastra and Bruni (18). Those investigators demonstrated that pretreatment of mice and rabbits with liposomes causes a significant reduction in their TNF response to LPS. Interestingly, phospholipid mixtures enriched with phosphatidylserine and phosphatidylethanolamine were the most effective. The liposomes used in the present studies contained 5% phosphatidylserine. While TNF generally augments the microbicidal activity of phagocytic cells and enhances bacterial clearance (30), the production of excessive TNF during overwhelming infection correlates with mortality, and interventions designed to prevent its release or abrogate its action lead to improved outcomes (for a review, see reference 27). Thus, depending on the clinical scenario, the data suggest that the specific lipid composition of the liposome may warrant consideration when these drug delivery systems are being designed.

This work was supported by grants from the Medical Research Council of Canada and the Defense and Civil Institute of Environmental Medicine.

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