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RESEARCH PAPER

Evaluation of Microspheres Containing Cytokine Neutralizing Antibodies in Endotoxemia

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ABSTRACT

Albumin microspheres are efficient carriers for delivering therapeutic agents to macrophages. In response to endotoxin, macrophages release tumor necrosis factor alpha (TNFα) and interleukin-1-beta (IL-1β). Blocking the effects of TNFα and IL-1β decreased lethality due to endotoxin-induced shock. In this study, we compared the efficacy of the microsphere form of TNFα and/or IL-1β neutralizing antibodies (NAs) with the free form of TNFα and/or IL-1β NA in preventing lethality due to endotoxemia and evaluated the duration of blockade by the microsphere form of TNFα and/or IL-1β NA on endotoxin-induced cytokine release. The results indicate that the microsphere form of TNFα and/or IL-1β NA protected 80% of the rats from lethal endotoxemia, while none of the rats that received the free form of TNFβ and/or IL-1β NA survived longer than 48 hr. The microsphere form of TNFα and/or IL-1β NA attenuated endotoxin-induced cytokine release more potently than the free form of TNFα and/or IL-1β NA in vivo. In vitro, the microsphere form of TNFα and/or IL-1β NA blocked endotoxin-induced cytokine release for at least 24 hr. Higher efficacy of the microsphere form of NA in reducing mortality and blocking cytokine release makes it more therapeutically advantageous than the free form of NA in the treatment of lethal endotoxemia.

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INTRODUCTION

Albumin microspheres are biodegradable, nonantigenic carrier particles with favorable characteristics for delivering immunomodulating drugs to phagocytic cells. Therapeutic agents inside the microspheres can be protected from in vivo degradation and dilution, thereby increasing efficacy of the drugs inside the microspheres. In addition, microspheres are readily taken up by phagocytic cells (macrophages) after systemic, pulmonary, and peritoneal administration, and the microencapsulated drug can be delivered into the macrophage without affecting other cell types, therefore reducing toxicity to nonphagocytic cells in the body (1–3). Albumin microspheres are used to deliver diagnostic agents to the reticuloendothelial system in clinical imaging and are currently being evaluated for delivering therapeutic agents like azidothymidine (AZT), vaccine adjuvants, and antibodies to macrophages (3,4–7).

Microspheres are similar to liposomes in their mechanism of delivering therapeutic agents to macrophages. The liposome form of therapeutic agents has been shown to be more efficacious than the free form in the treatment of bacterial, viral, and protozoal infections in experimental studies (8–10). Recently, it was shown that microspheres containing neutralizing antibodies (NAs) to tumor necrosis factor alpha (TNFα) and interleukin-1-beta (IL-1β) were more efficient than the free form of TNFα and IL-1β in blocking endotoxin-induced cytokine release in an in vitro whole blood model in rats (5). Endotoxin, a lipopolysacchride (LPS) component of the outer membrane of gram-negative bacteria, causes sepsis syndrome and septic shock in animals and humans. Activated macrophages release cytokines such as TNFα and IL-1β in response to endotoxin, and these cytokines are speculated to cause septic shock syndrome (11–13). The free form of TNFα NAs and interleukin-1 receptor antagonist (IL-1ra) decreased lethality induced by endotoxemia in animals, but not in humans (14–18). We hypothesize that microencapsulated forms of TNFα and IL-1β NA may be more efficacious than the free form of TNFα and/or IL-1β NA in vivo.

The purpose of this study was to compare the in vivo efficacy of free and microencapsulated forms of TNFα and/or IL-1β NA in protecting rats from lethal endotoxemia. In addition, we also evaluated the duration of blockade by microspheres containing NA to TNFα and IL-1β on endotoxin-induced cytokine release in vitro. The results indicate that microspheres containing TNFα and/or IL-1β NA protected 80% of the rats from lethal endotoxemia compared to 0% survival in the rats treated with the free form of TNFα and/or IL-1β NA, and the microspheres containing TNFα and/or IL-1β NA were capable of attenuating endotoxin-induced cytokine release for a minimum of 24 hr in vitro.

MATERIALS AND METHODS

Chemicals and Reagents

Human albumin, murine albumin, and Span 85 were obtained from Sigma Chemical Company (St. Louis, MO). Monoclonal antihuman TNFα NA, monoclonal antihuman IL-1β NA, polyclonal antimurine TNFα NA, polyclonal antimurine IL-1β NA, human TNFα and IL-1β, murine TNFα and IL-1β were obtained from R&D Systems (Minneapolis, MN). Tween 20 was obtained from Fisher Scientific (Fair Lawn, NJ), and endotoxin 0113 was obtained from Associates of Cape Cod (Woods Hole, MA).

Microsphere Preparation

Microspheres containing human albumin (blank), murine albumin (blank), monoclonal antihuman TNFα NA, monoclonal anti-human IL-1β NA, polyclonal antimurine TNFα NA, and polyclonal antimurine IL-1β NA were prepared by an emulsion method as described in an earlier paper (5). Briefly, albumin microspheres were prepared by dissolving equal amounts (by weight) of NA and albumin (1:1) or only albumin (for blanks) in phosphate-buffered saline and emulsifying with olive oil. Dispersion of the solution containing albumin and NA in olive oil resulted in the formation of microspheres. After 10 min of emulsification, the albumin was cross-linked using glutaraldehyde. The emulsion was stirred with a magnetic stirrer set at 200 rpm for 6 hr at 10°C–15°C and then centrifuged at 5000 rpm for 30 min to separate the microspheres. The microspheres were washed with hexane to remove the olive oil. The microspheres were sized by sequential reduction with sequential membrane filters to obtain microspheres that were greater than 0.75 μm in size and less than 1 μm in size. The microspheres were freeze-dried for 5 hours and stored at −70°C. The

microspheres were resuspended in saline containing 0.1% Tween 20 before use. The concentration of drug in each milligram of microspheres was 0.5 mg (or 50% by weight).

Experiment 1: Comparison of Neutralizing Antibodies in Free and Microsphere Form for Protecting Animals Against Lethal Endotoxemia

Fischer rats weighing 100–150 gm were housed in a controlled environment with a 12-hr light-dark cycle and were provided with free access to food and water. All the animals (50 total) in the study were given an intravenous injection of 15 mg/kg endotoxin 0113 obtained from *Escherichia coli*. Then, the animals were divided into various treatment groups as follows: (*a*) 10 animals were given no further treatment, (*b*) 5 animals were treated with intravenous saline, (*c*) 5 animals were treated intravenously with blank microspheres containing murine albumin, (*d*) 5 animals were treated intravenously with 1 mg/kg of antimurine TNFα NA in free form, (*e*) 5 animals were treated intravenously with 1 mg/kg of antimurine TNFα NA in microsphere form (present in 2 mg of microspheres), (*f*) 5 animals were treated intravenously with 2 mg/kg of antimurine IL-1β NA in free form, (*g*) 5 animals were treated intravenously with 2 mg/kg of antimurine IL-1β NA in microsphere form (present in 4 mg of microspheres), (*h*) 5 animals were treated intravenously with 1 mg/kg of antimurine TNFα NA in free form and 2 mg/kg of antimurine IL-1β NA in free form, and (*i*) 5 animals were treated intravenously with 1 mg/kg of antimurine TNFα NA in microsphere form (present in 2 mg of microspheres) and 2 mg/kg of antimurine IL-1β NA in microsphere form (present in 2 mg of microspheres). The rats were anesthetized with ether, and blood samples from the tail vein were collected at 0, 2, 6, 24, and 48 hr for measurement of plasma TNFα and IL-1β levels using an enzyme-linked immunosorbent assay (ELISA). The ELISA procedure was similar to that used to measure human cytokines (19,20), except that murine cytokines and corresponding antibodies were used instead of human cytokines and corresponding antibodies.

Experiment 2: Evaluation of Microspheres Containing Neutralizing Antibodies to Tumor Necrosis Alpha and Interleukin-1-Beta on Duration of Attenuation In Vitro

Whole blood was collected in tubes containing ethylenediaminetetraacetate (EDTA) from 10 healthy human volunteers after obtaining their informed consent. The study was approved by the Institutional Review Board of Mercer University. A baseline plasma sample was taken for cytokine measurement, and the blood was divided into seven aliquots for the study. The first aliquot was treated with blank microspheres containing human albumin, and plasma samples were collected at 2 and 24 hr for measurement of TNFα and IL-1β levels. The second, third, and fourth aliquots were pretreated for 2, 6, and 24 hr, respectively, with blank microspheres containing human albumin, and the fifth, sixth, and seventh aliquots were pretreated for 2, 6, and 24 hr, respectively, with microspheres containing 2 μg of antihuman TNFα NA (present in 4 μg of microspheres) and 4 μg of anti-human IL-1β NA (present in 8 μg of microspheres) for each milliliter of blood.

All the aliquots were incubated at 37°C under an atmosphere containing 5% CO_2. After 2, 6, and 24 hr of pretreatment with microspheres, endotoxin (100 ng/ml) was added to all aliquots except the first aliquot, and the aliquots were incubated at 37°C under an atmosphere containing 5% CO_2 for 24 hr. Plasma samples were collected at 2 and 24 hr by centrifugation for 10 min, and TNFα and IL-1β levels were measured in duplicate using an ELISA (19,20).

Statistical Analysis

The survival data from experiment 1 were analyzed using the one-sided Fisher's exact probability test, and the significant differences ($P < .01$) in the survival rate for 5 days or more were determined. The cytokine data from experiment 1 are expressed as mean ± standard error of the mean (SEM). Due to the loss of animals to endotoxin-induced death in experiment 1, the statistical significance for difference in cytokine levels at 2 and 6 hr was analyzed using an unpaired two-tailed *t* test. The data from experiment 2 were analyzed using multifactorial analysis of variance (ANOVA) followed by post hoc analysis with Dunn's multiple comparison test.

RESULTS

Experiment 1

The survival rates after lethal endotoxemia in rats after various interventions are shown in Table 1. The rats that received endotoxin alone, endotoxin plus saline, endotoxin plus blank microspheres, endotoxin plus the free form of TNFα NA and/or the free form of IL-1β NA died within 48 hr of treatment. On the other hand, 80% of the rats that received endotoxin plus the microsphere

Table 1

Effect of Treatment with Free and Microsphere Form of Neutralizing Antibodies to TNFα and/or IL-1β on Survival Rate in Rats with Lethal Endotoxemia

Treatments (*n*)	Percentage Survival (%)[a], Hours After Endotoxin					
	0	24	48	72	96	120
Endotoxin (10)	100	30	0	0	0	0
Endotoxin + saline (5)	100	0	0	0	0	0
Endotoxin + Blk. MC (5)	100	20	0	0	0	0
Endotoxin + free TNFα NA (5)	100	40	0	0	0	0
Endotoxin + TNFα MCNA solution (5)	100	100	80	80	80	80[b]
Endotoxin + free IL-1β NA (5)	100	20	0	0	0	0
Endotoxin + IL-1β MCNA (5)	100	100	80	80	80	80[b]
Endotoxin + free combo NA (5)	100	60	0	0	0	0
Endotoxin + combo MCNA (5)	100	100	80	80	80	80[b]

n, number of animals in each group; Blk. MC, blank microspheres; NA, Neutralizing antibodies; MCNA, microencapsulated cytokine neutralizing antibodies; combo, combination of TNFα and IL-1β neutralizing antibodies.

[a] Survival rate is expressed as the percentage of animals that were alive at each time point. Animals that were alive for 5 days or more were considered survivors.

[b] Statistically significant increase in survival rate at $P < .01$ when compared to the endotoxin only group.

form of TNFα NA and/or the microsphere form of IL-1β NA survived for 5 days or more ($P < .01$).

TNFα levels were significantly increased by endotoxin from 2 to 6 hr and decreased toward baseline levels by 24 hr (Table 2). The free form of TNFα NA and the free form of the combination TNFα/IL-1β NA attenuated endotoxin-induced TNFα levels significantly ($P < .05$) at 2 and 6 hr, while the free form of IL-1β NA had a trend for attenuation of endotoxin-induced TNFα levels at 2 and 6 hr (Table 2). Similarly, treatment with the microsphere form of TNFα NA and/or IL-1β NA significantly ($P < .05$) attenuated endotoxin-induced TNFα levels at 2 and 6 hr (Table 2). The attenuation of endotoxin-induced TNFα levels by the microsphere form of TNFα NA and/or IL-1β NA (0.5–5%) was significantly ($P < .05$) greater than that caused by the free form of TNFα and/or IL-1β NA (39–83%).

An interesting observation was that endotoxin-induced TNFα levels at 24 hr were considerably higher when treated with the free form of TNFα and/or IL-1β NA compared to no treatment, saline treatment, or blank microsphere treatment. In contrast to TNFα levels, IL-1β levels increased gradually over 24 hr after endotoxin challenge (Table 3). Treatment with the free form of TNFα NA and/or IL-1β NA did not attenuate endotoxin-induced IL-1β levels, but treatment with the microsphere form of TNFα NA and/or IL-1β NA significantly ($P < .05$) attenuated endotoxin-induced IL-1β levels at 2 and 6 hr (Table 3).

Experiment 2

Blank microsphere treatment did not significantly increase TNFα and IL-1β levels compared to baseline levels. Pretreatment (2, 6, and 24 hr) with blank microspheres caused endotoxin-induced TNFα release at 2 hr and IL-1β release at 2 and 24 hr to increase as the length of pretreatment time with blank microspheres was increased from 2 to 24 hr (Figs. 1 and 2, $P < .05$), but a similar pattern of increase was not seen with TNFα release at 24 hr after endotoxin. On the other hand, pretreatment with microspheres containing NA to TNFα and IL-1β for 2, 6, and 24 hr completely attenuated endotoxin-induced TNFα release at 2 hr (Fig. 1, $P < .05$) and IL-1β release at 2 hr and 24 hr (Fig. 2, $P < .05$).

DISCUSSION

This study demonstrated that the microsphere form of cytokine NA was more efficacious than the free form of cytokine NA in the treatment of lethal endotoxemia.

Table 2

TNFα Levels After Treatment with Free and Microsphere Form of Neutralizing Antibodies to TNFα and/or IL-1β in Lethal Endotoxemia

Treatments	TNFα Levels (pg/ml) as Mean ± SEM (*n*)				
	Baseline	2 hr	6 hr	24 hr	48 hr
Endotoxin	<20 (10)	4176 ± 357 (10)	2322 ± 263 (10)	27 ± 12 (3)	—
Endotoxin + saline	20 ± 3 (5)	3363 ± 402 (5)	2936 ± 271 (5)	—	—
Endotoxin + Blk. MC	<20 (5)	3635 ± 1069 (4)	2265 ± 342 (4)	114 (1)	—
Endotoxin + free TNFα NA	<20 (5)	1997 ± 113[a,b] (5)	1588 ± 126[a,b] (5)	859 ± 114 (2)	—
Endotoxin + TNFα MCNA	<20 (5)	176 ± 45[a,b,c] (5)	63 ± 15[a,c,d] (5)	<20 (5)	<20 (4)
Endotoxin + free IL-1β NA	22 ± 4 (5)	2793 ± 204 (5)	2299 ± 227 (5)	1012 (1)	—
Endotoxin + IL-1β MCNA	<20 (5)	158 ± 39[a,c,d](5)	85 ± 9[a,c,d] (5)	30 ± 2 (5)	<20 (4)
Endotoxin + free combo NA	20 ± 4 (5)	2413 ± 221 (5)	1137 ± 134[a,b] (5)	644 ± 49 (3)	—
Endotoxin + combo MCNA	<20 (5)	<20[a,c,d] (5)	<20[a,c,d] (5)	<20 (5)	<20 (4)

SEM, standard error of the mean; *n*, number of animals; Blk. MC, blank microspheres; NA, neutralizing antibodies; MCNA, microencapsulated cytokine neutralizing antibodies; Combo, combination of TNFα and IL-1β neutralizing antibodies.

[a] Statistical significance at $P < .05$ using a two-tailed *t* test.

[b] Significant statistical significance at $P < .05$ compared to endotoxin plus saline group.

[c] Significant statistical significance at $P < .05$ compared to endotoxin plus Blk. MC group.

[d] Significant statistical significance at $P < .05$ compared to endotoxin plus the corresponding free form of neutralizing antibody group.

Treatment with the free form did not protect the rats from lethality due to endotoxemia, while equivalent doses in the microsphere form protected 80% of the rats from lethality. Other researchers have shown that the free form of 15 mg/kg anti-TNFα monoclonal antibody and 100 mg/kg IL-1ra protected animals from the lethality due to endotoxemia (14–18). However, this is the first study to show that a low dose of 1 mg/kg anti-TNFα antibody in the microsphere form can protect animals from the lethality due to endotoxemia. The higher efficacy of the microsphere form of TNFα and/or IL-1β NA in the endotoxemia model of septic shock can be attributed to

Table 3

IL-1β Levels After Treatment with Free and Microsphere Form of Neutralizing Antibodies to TNFα and/or IL-1β in Lethal Endotoxemia

Treatments	IL-1β Levels (pg/ml) as Mean ± SEM (*n*)				
	Baseline	2 hr	6 hr	24 hr	48 hr
Endotoxin	<20 (10)	53 ± 4 (10)	173 ± 10 (10)	279 ± 4 (3)	—
Endotoxin + saline	27 ± 3 (5)	35 ± 3 (5)	168 ± 6 (5)	—	—
Endotoxin + Blk. MC	<20 (5)	38 ± 3 (4)	148 ± 2 (4)	293 (1)	—
Endotoxin + free TNFα NA	<20 (5)	37 ± 5 (5)	174 ± 5 (5)	307 ± 11 (2)	—
Endotoxin + TNFα MCNA	<20 (5)	12 ± 3[a,b,c] (5)	<20[a] (5)	40 ± 2 (5)	54 ± 4 (5)
Endotoxin + free IL-1β NA	25 ± 3 (5)	40 ± 3 (5)	153 ± 13 (5)	316 (1)	—
Endotoxin + IL-1β MCNA	<20 (5)	31 ± 4 (5)	26 ± 3[a] (5)	35 ± 4 (5)	38 ± 1 (4)
Endotoxin + free combo NA	<20 (5)	35 ± 3 (5)	142 ± 12 (5)	179 ± 30 (3)	—
Endotoxin + combo MCNA	<20 (5)	<20[a] (5)	<20[a] (5)	33 ± 2 (5)	37 ± 1 (4)

SEM, standard error of the mean; *n*, number of animals; Blk. MC, blank microspheres; NA, neutralizing antibodies; MCNA, microencapsulated cytokine neutralizing antibodies; Combo, combination of TNFα and IL-1β neutralizing antibodies.

[a] Statistical significance at $P < .05$ using a two-tailed *t* test.

[b] Significant statistical significance at $P < .05$ compared to endotoxin plus Blk. MC group.

[c] Significant statistical significance at $P < .05$ compared to endotoxin plus the corresponding free form of neutralizing antibody group.

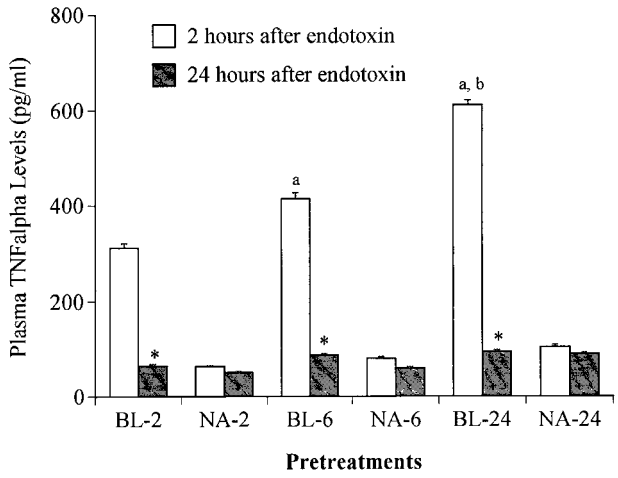

Figure 1. Effect of pretreatment with blank microspheres or microspheres containing neutralizing antibodies to TNFα and IL-1β for 2, 6, and 24 hr on endotoxin-induced TNFα release in the whole blood model (BL, blank microspheres; NA, microspheres containing neutralizing antibodies to TNFα and IL-1β). *Significant ($P < .05$) blockade of endotoxin-induced TNFα release at 2 hr compared to the corresponding blank microsphere pretreatment group. [a]Significant ($P < .05$) increase in endotoxin-induced TNFα release at 2 hr compared to the BL-2 at 2 hr. [b]Significant ($P < .05$) increase in endotoxin-induced TNFα release at 2 hr compared to the BL-6 at 2 hr.

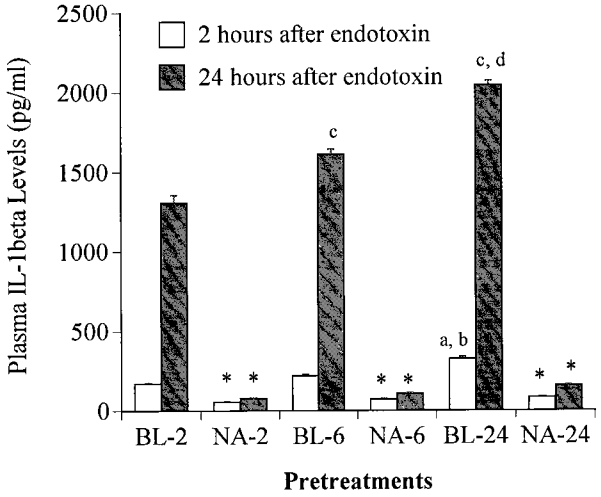

Figure 2. Effect of pretreatment with blank microspheres or microspheres containing neutralizing antibodies to TNFα and IL-1β for 2, 6, and 24 hr on endotoxin-induced IL-1α release (BL, blank microspheres; NA, microspheres containing neutralizing antibodies to TNFα and IL-1β). *Significant ($P < .05$) blockade of endotoxin-induced IL-1β release at 2 and 24 hr compared to the corresponding blank microsphere pretreatment group. [a]Significant ($P < .05$) increase in endotoxin-induced IL-1β release at 2 hr compared to the BL-2 at 2 hr. [b]Significant ($P < .05$) increase in endotoxin-induced IL-1β release at 2 hr compared to the BL-6 at 2 hr. [c]Significant ($P < .05$) increase in endotoxin-induced IL-1β release at 24 hr compared to the BL-2 at 24 hr. [d]Significant ($P < .05$) increase in endotoxin-induced IL-1β release at 24 hr compared to the BL-6 at 24 hr.

intracellular delivery of cytokine antagonists to monocyte/macrophages. Macrophages are the major source of endotoxin-induced cytokine release in animals and humans (11–13,21–23).

The large increase in TNFα levels and a smaller increase in IL-1β levels by endotoxin seen in this study have been shown by others in rodents and human volunteers (24,25). However, this is the first study to show that anti-IL-1β antibodies can increase survival and suppress TNFα release in lethal endotoxemia. The attenuation of TNFα levels by IL-1β NA and vice versa seen in this study reaffirms that these two cytokines modulate each other. The microsphere form of TNFα and/or IL-1β NA attenuated TNFα and IL-1β levels to a much greater extent than the free form of TNFα and/or IL-1β NA in the endotoxemia model. Similar results have been shown in vitro, in which the microsphere form of TNFα and IL-1β NA attenuated endotoxin-induced TNFα and IL-1β levels at least 1.5 to 2 times better than the free form of TNFα and IL-1β NA (5).

We speculate that the increased efficacy of the microsphere form of cytokine NA in the attenuation of cytokine levels might translate to the increased survival rate seen in the endotoxemia model of septic shock. On the other hand, in this study the combination of TNFα and IL-1β NA in the microsphere form protected the same percentage of rats (80%) from lethality due to endotoxemia as well as either one alone did. Russell and Colleagues (26) showed that inhibition of both TNFα and IL-1β was superior to either one alone in increasing survival and organ function after lethal endotoxemia in rodents. The difference in the results between these two studies could be due to the difference in the mechanism of action of the cytokine antagonists used. Russell and colleagues (26) used the free form of IL-1ra and polyethylene-glycol-linked dimer of the type I soluble receptor of TNF, while in this study the microsphere form of NA to TNFα and IL-1β was used.

The results of the in vitro endotoxin study indicate that the microsphere form of TNFα and IL-1β NA can block endotoxin-induced cytokine release for at least 20 hours if the microsphere form of NA was administered

prior to the endotoxin challenge. It has been shown that a single infusion of the free from of TNFα NA in patients with septic shock has a half-life of 52 hr (27). In addition, the microsphere form of other therapeutic agents like AZT has been shown to release the agent slowly as the microsphere matrix degraded slowly over 2 days inside the macrophage, providing a sustained effect (4). We speculate a similar sustained effect with the microspheres containing cytokine neutralizing antibodies. The probable increased duration of action and higher efficacy of the microsphere form (resulting in the use of lower doses) make it therapeutically advantageous over the free form.

CONCLUSIONS

Albumin microspheres can be used to target therapeutic agents to macrophages as they are readily phagocytosed by macrophages both in vitro and in vivo (1,7). In vitro radio-labeled uptake experiments carried out in our laboratory show that approximately 25% of the microspheres are phagocytosed by macrophages within 60 min (28). Once inside the macrophage, we speculate that the NAs are released inside the macrophages/monocytes by lysosomal-enzyme-mediated breakdown of the microsphere matrix and/or by leakage of NA out of the microspheres. It has been shown that the cytosol of macrophages contains cytokines after LPS stimulation (29). We speculate that the NA may self-attenuate and attenuate other cytokines in the cytosol via simple neutralization and/or by affecting the transcription/translational process in the synthesis of these cytokines. A small amount of the whole NA or fragments of it may also be extruded from the cell to neutralize the cytokines present extracellularly. Blocking cytokines intracellularly with the microsphere form of cytokine NA may be more efficacious than blocking cytokines extracellularly with the free form of NA.

Finally, survival after lethal endotoxemia can be significantly improved by low doses of microspheres containing TNFα and IL-1β NA. The microsphere form of cytokine inhibitors can be therapeutically beneficial in the treatment of septic shock syndrome because the form is highly efficacious and has a long duration of action with minimal side effects.

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