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RESEARCH PAPER

Macrophage Depletion by Albumin Microencapsulated Clodronate: Attenuation of Cytokine Release in Macrophage-Dependent Glomerulonephritis

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ABSTRACT

*A macrophage plays an important role in mediating the inflammatory response. Cytokines released by activated macrophages contribute to inflammation in glomerulonephritis (GN). Clodronate, a biphosphonate, causes macrophage depletion when administered in an encapsulated form in liposomes. We used albumin as the polymer matrix to microencapsulate clodronate to the microspheres (MS) in the 1-μm size range. The purpose of this study was to (*a*) determine macrophage depletion by clodronate MS, (*b*) determine the effect of clodronate MS on endotoxin-induced cytokine release in vitro, and (*c*) assess the effect of clodronate MS on macrophage infiltration in experimental antiglomerular basement membrane nephritis. Macrophage depletion by clodronate MS was assessed by staining for the*

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ED1 marker. The results indicate greater than 95% depletion of macrophages from the spleen, liver, kidney, and blood. In the whole blood model, clodronate MS attenuated endotoxin-induced tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) release, and the attenuation by the microencapsulated form of clodronate was also more effective than the free (solution) form of clodronate. Macrophage infiltration into the glomerulus in experimental GN was also blocked very effectively by pretreatment with clodronate MS. In conclusion, macrophage depletion by clodronate MS may be beneficial in reducing cytokine release and renal damage in GN.

Key Words. *Albumin microspheres; Clodronate; Glomerulonephritis; Interleukin-1 beta; Macrophage; Tumor necrosis factor alpha*

INTRODUCTION

The macrophage plays an important role in the inflammatory process through the release of cytokines, chemokines, and other substances. The role of the macrophage in various inflammation-mediated disease states can be evaluated by depletion of macrophages with clodronate (1). Clodronate, a biphosphonate, is a potent inhibitor of osteoclast-mediated bone reabsorption and is used clinically to treat metabolic bone diseases (2). Clodronate in free (solution) form has little effect on macrophage function following systemic administration (3). However, liposomes containing clodronate are readily phagocytosed by macrophages and cause depletion of macrophages in the liver, spleen, lymph nodes, and peritoneal cavity and of monocytes in the systemic circulation (3–6). We have developed a method of microencapsulation of clodronate using albumin that has several advantages over the use of liposomes. Albumin can be used as the biocompatible polymer matrix to form microspheres (MS) of varying size that have greater stability and ease of preparation compared to liposomes. Albumin is a biodegradable, nontoxic substance that has a high efficiency of encapsulation. The purpose of this investigation was to determine if albumin MS containing clodronate (*a*) will produce systemic macrophage depletion, (*b*) have an effect on tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) release induced by endotoxin in vitro, and (*c*) have an effect on macrophage infiltration in experimental glomerulonephritis (GN) in rats. The results indicate that clodronate MS effectively depleted macrophages, attenuated endotoxin-induced TNF-α and IL-1β release, and blocked experimental GN-induced macrophage infiltration into the glomerulus.

METHODS

Microencapsulation of Clodronate Containing Albumin MS

Equal amounts of albumin (Sigma Chemical Company, St. Louis, MO) and clodronate (Boehringer Mannheim Laboratory, Germany) were dissolved in the aqueous phase and homogenized in olive oil for 10 min to form an emulsion. The emulsion was then sonicated for 10 min in a Branson Sonifier at medium setting (Fisher Scientific, Pittsburgh, PA). Glutaraldehyde (Sigma Chemical Company) was then added to cross link albumin and stabilize the MS. The olive oil was washed off with the aid of hexane followed by centrifugation. The MS were dried in a vacuum desiccator. Sizing of the MS to 1 μm was achieved using sequential nylon filters of the high-performance liquid chromatography (HPLC) type. Blank MS were prepared in the manner described above without clodronate.

Comparison of In Vitro Efficacy of Free Form and Microsphere Form of Clodronate in Rat Whole Blood Model

Blood from six to seven Fisher rats (F-344) weighing 200–250 grams (obtained from Harlan Sprague-Dawley) was collected via cardiac puncture and pooled for each *n*. For each milliliter of blood, 10 μl of 15% ethylenediaminetetraacetate (EDTA) solution was added to prevent clotting. The blood was aliquoted, and to each milliliter, 25, 50, and 100 μg of free clodronate in saline or 50, 100, and 200 μg of clodronate MS (equivalent to 25, 50, and 100 μg of free clodronate, respectively, since the albumin : clodronate ratio in the microencapsulated clodro-

nate formulation was 1:1) were added. An aliquot of blood from each rat was also treated with 50 μl of saline or 400 μg of blank MS. Two hours later, 100 ng/ml of endotoxin was added, and the blood samples were incubated for 24 hr in an atmosphere of 5% CO_2 at 37°C. Plasma samples were collected at baseline, 2, 4, 6, and 24 hr by centrifugation at 1000× g for 10 min, for measurement of TNF-α and IL-1β using a modified enzyme-linked immunosorbent assay (ELISA) procedure developed in our laboratory as described below.

Measurement of TNF-α and IL-1β by ELISA

TNF-α and IL-1β were analyzed by an assay developed in our laboratory by modification of the ELISA method to measure human TNF-α (7) and human IL-1β (8). Briefly, the microtiter plates were coated with 100 ml of murine TNF-α or IL-1β (10–5000 pg/ml) for 24 hr. The plates were then washed, and 100 μl of goat anti-murine TNF-α antibody or IL-1β antibody (R&D Systems, Minneapolis, MN) were added into each well and incubated for 1 hr at 37°C. The plates were washed after the incubation, and 100 μl of rabbit antigoat immunoglobulin G (IgG) antibody linked to horse radish peroxidase (R&D Systems) were added. The plates were washed again, the enzymatic activity was detected with *o*-phenylenediamine dihydroxy hydrochloride, and the absorbance was measured at 492 nm on an ELISA plate reader. A linear relationship with a correlation coefficient less than 0.95 was observed for the standard curve (range 10–5000 pg/ml). The cross-reactivity with other cytokines tested (such as murine IL-2, IL-6, and IL-8) was less than 10%.

Macrophage Depletion by Clodronate in Healthy Rats and Rats with Anti-GBM GN

Anti-GBM (anti–glomerular basement membrane) globulin was raised in sheep by repeated immunization with a membrane fraction of rat kidney in Freund's complete adjuvant (FCA; Sigma Chemical Co.). The sheep serum was heat decomplemented and absorbed twice against rat red blood cells (10% by volume). A globulin fraction was prepared by precipitation with ammonium sulfate at a final concentration of 50% and was extensively dialyzed against phosphate buffered saline.

Glomerulonephritis was initiated by intravenous injection of sheep antirat GBM globulin at a dose of 100 μg/g body weight to male Sprague-Dawley rats weighing 100–150 g, obtained from Central Animal Services (Monash University, Clayton, Victoria, Australia). Forty-eight hours prior to initiation of anti-GBM GN, one group of rats received 5 mg of clodronate MS (assumed to contain not more than 50% clodronate by weight), and the other group received no clodronate treatment. A group of healthy normal rats that did not receive any anti-GBM GN was used as controls. Seventy-two hours after anti-GBM injection, all the rats (including the healthy control rats) were sacrificed, and tissue samples of the spleen, liver, and kidney were obtained from each rat. The tissue samples were then fixed in periodate lysine paraformaldehyde for 4 hr, washed in 7% sucrose solution, and then frozen in liquid-nitrogen-cooled isopentane. The frozen tissue was sliced into 4-mm sections in a cryostat. Tissue sections were stained using a three-layer immunoperoxidase technique. A mouse monoclonal antibody against rat ED1, a pan-macrophage marker that reacts with the cytoplasmic antigen (9), was the primary antibody added. This was followed by a second layer of rabbit antimouse IgG globulin at a concentration of 1:100 (Dako, Glostrup, Denmark). This was followed by a peroxidase-conjugated mouse immunoglobulin (Dako) at a concentration of 1:100. Sections were then incubated with diamino benzadine (Sigma Chemical Company), and counterstained with Harris haemotoxylin. The number of macrophages in the spleen was measured by counting ED1-positive cells in 10 × 1 mm^2 red pulp areas and averaged as cells/mm^2. The number of Kupffer cells in the liver was measured by counting ED1-positive cells in 10 × 1 mm^2 liver cord areas and averaged as cells/mm^2. Macrophages in circulation were calculated as the percentage of the circulating leukocytes.

STATISTICAL ANALYSIS

The data obtained from the study using the whole blood model were analyzed using an multivariate analysis of variance (ANOVA), followed by Tukey's HSD for post hoc analysis. The data from the macrophage depletion study were analyzed using an unpaired *t* test.

RESULTS

The effect of clodronate on endotoxin-induced TNF-α and IL-1β release is shown in Figs. 1 and 2, respectively. Presence of blank MS did not significantly affect endotoxin-induced TNF-α and IL-1β release. Low (25 μg/ml) and medium (50 μg/ml) doses of free clodronate did not alter endotoxin-induced TNF-α and IL-β release, but a higher dose (100 μg/ml) of free clodronate showed a

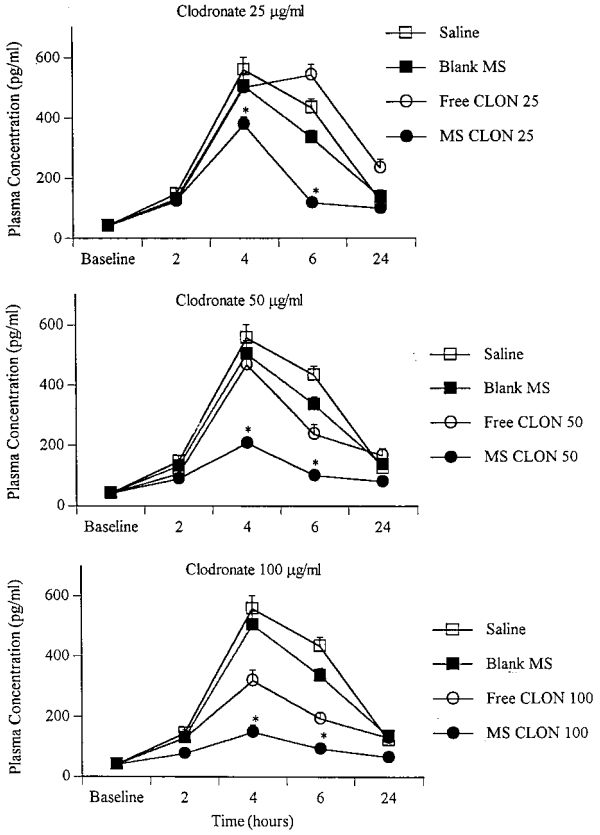

Figure 1. Effect of free form of clodronate (CLON) and microspheres (MS) of CLON on endotoxin-induced TNF-α levels in the rat whole blood model. To each milliliter of blood, 25, 50, and 100 μg of free clodronate in saline or 50, 100, and 200 μg of clodronate MS (equivalent to 25, 50, and 100 μg of free clodronate, respectively) in saline were added. In all groups, the saline group received 50 μl of saline, and the blank MS group received 400 μg of blank MS for each milliliter of blood. Two hours later, 100 ng/ml of endotoxin was added, and the blood was incubated for 24 hr in an atmosphere of 5% CO_2 at 37°C. Plasma levels after endotoxin challenge are shown in this figure. *MS form of CLON-attenuated endotoxin-induced TNF-α levels significantly better than the free form of CLON at $P < .05$ level.

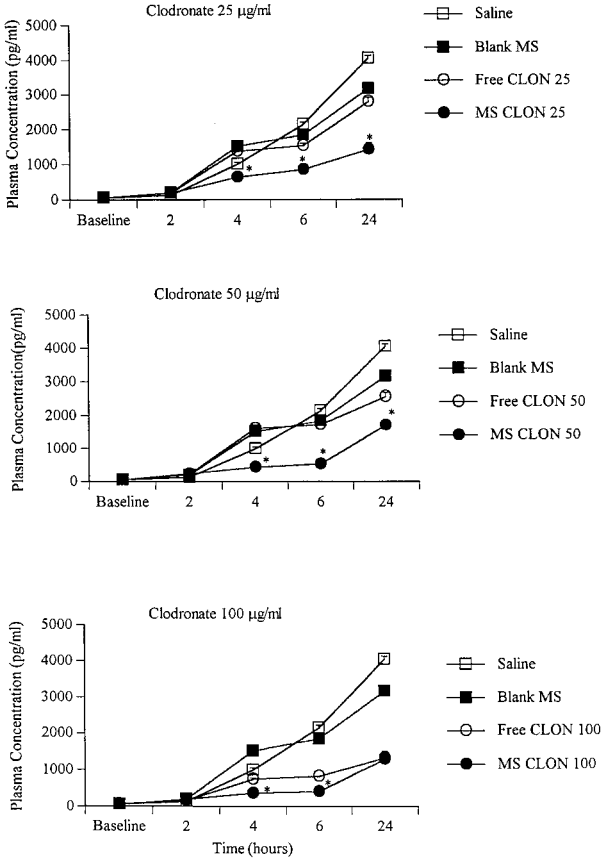

Figure 2. Effect of free form of clodronate (CLON) and microspheres (MS) of CLON on endotoxin-induced IL-1β levels in the rat whole blood model. To each milliliter of blood, 25, 50, and 100 μg of free clodronate in saline or 50, 100, and 200 μg of clodronate MS (equivalent to 25, 50, and 100 μg of free clodronate, respectively) in saline were added. In all groups, the saline group received 50 μl of saline, and the blank MS group received 400 μg of blank MS for each milliliter of blood. Two hours later, 100 ng/ml of endotoxin was added, and the blood was incubated for 24 hr in an atmosphere of 5% CO_2 at 37°C. Plasma levels after endotoxin challenge are shown in this figure. *MS form of CLON-attenuated endotoxin-induced IL-1β levels significantly better than the free form of CLON at $P < .05$ level.

trend for attenuating endotoxin-induced TNF-α and IL-1β release. On the other hand, all doses of clodronate MS containing 25, 50, and 100 μg of equivalent free clodronate per milliliter significantly ($P < .05$) attenuated endotoxin-induced TNF-α and IL-1β release.

Tissue sections stained for ED1-positive macrophages demonstrate that there was a significant ($P < .001$) reduction of ED1-positive macrophages from the liver and spleen of rats that received clodronate MS compared to healthy control rats (Table 1; Fig. 3). There was also a significant reduction in circulating monocytes in peripheral blood (Table 1, $P < .001$). Similarly, kidney sections stained for ED1-positive macrophages show that there was no macrophage infiltration into the glomerulus of normal healthy kidneys (Fig. 4, panel A), and induction of anti-GBM GN caused ED1-positive macrophage infil-

Table 1

Effect of Clodronate MS on Macrophage Depletion in Rats with Anti-GBM GN

	ED1 Positive Macrophages	
Tissue	Untreated Controls	Clodronate MS
Spleen	280 ± 23 cells/mm^2	13 ± 12 cell/mm^{2,*}
Liver	38 ± 2.5 cells/mm^2	1.8 cells/mm^{2,*}
Blood	13.5 ± 1.5%[a]	1.2 ± 0.1%[a,*]

[a] Percentage of the total leucocytes.
* Statistical significance at $P < .001$.

tration (Fig. 4, panel B). Pretreatment with clodronate MS significantly reduced the macrophage infiltration in anti-GBM GN (Fig. 4, panel C).

DISCUSSION

Our study demonstrates that small doses of clodronate encapsulated in albumin are effective in depleting ED1-positive macrophages from the liver, spleen, kidney, and peripheral blood in rats. Clodronate MS also produced a prompt reduction in endotoxin-stimulated TNF-α and IL-1β release that was significantly greater than clodronate in free (solution) form and prevented macrophage infiltration into the glomerulus, which accumulate during experimental anti-GBM GN in rat.

Macrophage depletion has been proven to be a valuable tool in evaluating the contribution of the macrophage to the development of pathological conditions. Clodronate, a biphosphonate, has little effect on the viability of the macrophage in the free form, but encapsulated into liposomes or MS (as in this study), there was a transient depletion of the macrophage population within 24–48 hr (3,10,11). The depletion of macrophages by clodronate liposomes was shown to be caused by apoptosis-induced cell death (12). We speculate a similar mechanism of action for clodronate MS.

The reduction in endotoxin-induced TNF-α and IL-1β release after pretreatment with clodronate MS as seen in this study has also been shown by others using clodronate liposomes (13). It has also been shown that clodronate liposomes can attenuate cytokine gene expression in mice (14). In the whole blood model, we also demonstrated a greater reduction of endotoxin-induced cytokine release with clodronate MS when compared to clodronate in free form. There was nearly a complete inhibition of both TNF-α and IL-1β release at the highest dose of clodronate MS that contained not greater than 100 μg of free clodronate. The mechanism of action of clodronate MS is likely due to phagocytosis of the albumin MS containing clodronate in a fashion similar to liposomes, followed by the release of the clodronate intracellularly, which produces an inhibition of cytokine release due to death of macrophages (15). Inhibition of cytokine release by clodronate may be beneficial in the treatment of disease states characterized by proinflammatory cytokine release.

Previous studies have shown that macrophages have an important role in induction and progression of renal damage in GN (16–18). Hallmarks of GN are proteinuria and macrophage infiltration (19–21). The reduction in macrophage infiltration by clodronate MS in experimental GN has been previously shown by our group. We have shown that the anti-GBM–induced GN causes macrophage infiltration (8.2 cells/glomerular cross section), and treatment with clodronate MS prevented macrophage infiltration (2.2 cells/glomerular cross section) similar to that seen in this study (22). In addition, we have also shown that anti-GBM GN-induced proteinuria (43 mg/24 hr) can also be significantly reduced with clodronate MS (8.4 mg/24 hr) to the same level found in normal healthy rats (5.3 mg/24 hr) (22). Clodronate MS may be therapeutically beneficial by depleting macrophages in GN.

In conclusion, these studies demonstrate that albumin MS containing clodronate are an effective tool for total body depletion of macrophages in the rat. Depletion of macrophages by clodronate MS produced attenuation of proinflammatory cytokines and amelioration of experimental antiglomerular basement membrane GN, which has been demonstrated to be macrophage dependent. Transient depletion of macrophages may be a treatment modality for macrophage-dependent disease states.

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