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Cutting Edge: Nitrogen Bisphosphonate-Induced Inflammation Is Dependent upon Mast Cells and IL-1

John T. Norton,* Tomoko Hayashi,* Brian Crain,* John S. Cho,† Lloyd S. Miller,‡ Maripat Corr,‡ and Dennis A. Carson*

Nitrogen-containing bisphosphonates (NBPs) are taken by millions for bone disorders but may cause serious inflammatory reactions. In this study, we used a murine peritonitis model to characterize the inflammatory mechanisms of these agents. At dosages comparable to those used in humans, injection of NBPs into the peritoneum caused recruitment of neutrophils, followed by an influx of monocytes. These cellular changes corresponded to an initial increase in IL-1α, which preceded a rise in multiple other proinflammatory cytokines. IL-1R, IL-1α, and IL-1β were required for neutrophil recruitment, whereas other MyD88-dependent signaling pathways were needed for the monocyte influx. Mice deficient in mast cells, but not mice lacking lymphocytes, were resistant to NBP-induced inflammation, and reconstitution of these mice with mast cells restored sensitivity to NBPs. These results document the critical role of mast cells and IL-1 in NBP-mediated inflammatory reactions. The Journal of Immunology, 2012, 188: 2977–2980.

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lendronate (ALD), pamidronate (PMD), and zoledronate (ZLD) are Food and Drug Administration-approved nitrogen-containing bisphosphonates (NBPs) that are used as oral or i.v. treatments for osteoporosis and cancer-related bone disorders (1). Bisphosphonates bind to the bone surface, have a very long half-life, and thus can attain high local concentrations. They prevent osteoclast-mediated bone degradation through inhibition of the mevalonate pathway, which leads to altered protein prenylation (2). Several inflammatory reactions have been observed in patients treated with NBPs, of which osteonecrosis of the jaw is the most ominous (3, 4). NBPs can modulate immunity as a result of inhibition of the mevalonate pathway, leading to an accumulation of isopentenyl-5-pyrophosphate in monocytes, which then activate γδ T cells (5). However, γδ T cells have not been conclusively shown to be the major cause of NBP-induced inflammatory reactions in vivo. Pretreatment of mice with ALD increases IL-1 production in response to LPS (6, 7) and sensitizes cells to MyD88-dependent signaling (8); however, a more precise mechanism of NBP immune modulation has remained elusive. Hence, the goal of the studies presented in this article was to dissect the role of different cell types and mediators in NBP-induced inflammation.

Materials and Methods

Mice

C57BL/6, Rag1−/−, Beige (Ly5.2+/Ly5.1−/), Il1r1−/−, P2x7r−/−, and c-kir2.5−/− (W/Wv) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Nlys−/− mice were gifts from Dr. H. Hoffman (University of California San Diego, La Jolla, CA) (9), Capp1−/− and Myd88−/− mice were gifts from Dr. R. Flavell (Yale University, New Haven, CT) (10) and Dr. S. Akira (Osaka University, Osaka, Japan) (11), respectively, and were maintained on a C57BL/6 background at University of California San Diego. Pretty2 mice were a gift from Dr. Bruce Beutler (The Scripps Research Institute, La Jolla, CA) (12). Il1b−/− and Ilb−/− mice were obtained from Yoichiro Iwakura, University of Tokyo, Tokyo, Japan (13), and maintained on a C57BL/6 background. Mast cell engraftment to W/Wv mice was performed as described previously (14). All procedures and protocols were approved by the Institutional Animal Care and Use Committee.

Reagents

ALD, PMD, clodronate (CLD), cromolyn, compound 48/80, and lovastatin were purchased from Sigma-Aldrich (St. Louis, MO), and ZLD was purchased from AK Scientific (Mountain View, CA). Bisphosphonates were endotoxin free, as determined by the Limulus Amebocyte Lysate test (Charles River, Wilmington, MA). PBS was used as the vehicle control.

In vivo peritonitis experiments

Mice (8–12 wk old) were injected i.p. with 0.8 mg per mouse ALD or other bisphosphonates, as indicated. Peritoneal cells were recovered with 3 ml PBS; total cell numbers in peritoneal lavage fluids were determined with a Guava Personal Cytometer (Millipore, Danvers, MA), and cells were differentiated by Wright-Giemsa staining. The levels of cytokines and chemokines in serum and lavage fluid were analyzed with LumineX beads (Invitrogen, Carlsbad, CA). Cromolyn and compound 48/80 were i.p. injected, as previously described (14).

Mast cell preparation and in vitro assays

Bone marrow-derived mast cells (BMMC) were prepared in culture with 3 ng/ml IL-3 (BD Biosciences, San Jose, CA) for 6 wk (14). Cultures were confirmed to be >99% mast cells with toluidine blue staining and FACS analysis.
for FcεR1 and c-kit (BioLegend, San Diego, CA). Mesothelial cells were isolated as previously described (15) and were plated at a density of 10⁴ cells per well in 96-well plates. Mast cells (10⁵ cells per well) were added and cocultured with the mesothelial cells (1:10 ratio) in appropriate media with 50 ng/ml murine stem cell factor (Sigma-Aldrich) (16). IL-1α and IL-6 levels in the culture supernatants were measured by ELISA (BioLegend and BD Biosciences, respectively). Mast cell degranulation was measured by treating cells with 100 μM of the indicated compounds for 1.5 h and measuring β-hexosaminidase release in a colorimetric assay, as previously described (16).

**Statistics**

Statistical analyses for multiple comparisons were performed using one-way ANOVA with Dunnett’s or Bonferroni’s test, as indicated. p < 0.05 was considered significant.

**Results and Discussion**

**Characterization of NBP-induced peritonitis**

ALD, PMD, and ZLD all caused profound inflammation after injection into the peritoneal cavity of mice. In contrast, the non-NBP CLD did not cause peritonitis (Fig. 1A). The proinflammatory effect of NBPs, but not CLD, validated this model because NBPs can cause inflammatory side effects in humans, whereas CLD does not (2, 3). In subsequent experiments, ALD was used as a prototypical NBP to eluci-

**FIGURE 1.** Characterization of the murine peritonitis model induced by NBPs. (A) C57BL/6 mice (n = 5–6) were i.p. injected with different doses of the NBP’s PMD, ZLD, and ALD, as well as the non-NBP CLD. Infiltrating cells were recovered 3 d after administration. (B) Mice (n = 5) were injected i.p. with 0.8 mg/ml ALD, and peritoneal infiltrating cells were recovered at different time points. (C) The granulocyte populations were further differentiated into neutrophils, eosinophils, and mast cells on the basis of morphology. Data are represented as means ± SEM of pooled two independent experiments.

**FIGURE 2.** ALD causes a rapid increase in IL-1α and murine chemokine (C-X-C motif) ligand 1. Peritoneal lavage fluid and serum were collected at indicated time points from mice (n = 5) i.p. administered 0.8 mg ALD (black bars) or vehicle (white bars). The levels of chemokines and cytokines in lavage (A) and serum (B) were analyzed by Luminex bead assay. Data are represented as means ± SEM. *p < 0.05, **p < 0.01, compared with 0 h or vehicle controls (at the time point of significance with ALD) by one-way ANOVA with Dunnett’s test.

**FIGURE 3.** ALD requires mast cells to induce peritonitis. (A) WT C57BL/6 (n = 6 per group), Rag1−/− (n = 7 per group), beige (B6.BgJ, n = 5–6 per group), or Pretty2 (n = 3 per group) mice were injected with 0.8 mg ALD (black bar) or vehicle (white bar). The peritoneal lavage was collected after 3 d. (B) W/Wv mice, littermate WT mice, and W/Wv mice with reconstituted BMMCs received ALD (black) or vehicle (white). The peritoneal lavage was collected, and total cell number was determined. (C) ALD was injected in WT mice (n = 10–12 per group) treated with cromolyn (Cromo, n = 7–8 per group) or compound 48/80 (48/80, n = 5 per group). Peritoneal infiltrating cells were collected 3 d after ALD (black) or vehicle (white) injection. (D) Degranulation of BMMCs treated with 100 μM of each compound for 1.5 h measured by β-hexosaminidase released in supernatant (A₄⁰₅). For all panels, data are the pooled means ± SEM from two or three independent experiments. *p < 0.05 by one-way ANOVA with Dunnett’s test.
date the cell types mediating the inflammation (17). Intraperitoneal administration of as little as 91 nmol (29 μg) to a mouse induced an increase in leukocyte infiltration (Fig. 1A). Time course experiments demonstrated that neutrophils appeared in the peritoneal cavity after ALD injection, followed by a large-scale influx of monocytes (Fig. 1B) and neutrophils (Fig. 1C). The results indicated that two distinct phases of inflammation were caused by ALD (Fig. 1B, 1C). The increase in neutrophils correlated with a peak in IL-1α levels in the peritoneum, and in both IL-1α and murine chemokine (C-X-C motif) ligand 1, keratinocyte chemotactant, levels in the serum (p < 0.05; Fig. 2). The later influx of monocytes at 24–72 h correlated with a peak in MCP-1, MIP-1α, and IL-6 levels in the peritoneum and MCP-1 in the serum (Fig. 2).

**ALD-induced peritonitis is dependent upon mast cells**

Mice deficient in specific cell types were injected with ALD to determine which cells are responsible for the inflammatory effects of the drug. Rag1−/− mice lack mature lymphocytes, including γδ T cells (18), but developed ALD-induced peritonitis indistinguishable from that in wild-type (WT) mice (Fig. 3A). Similarly, Beige (B6.B129-Ptprca Pep3b/J) mice, which lack NK cells, had an unimpaired inflammatory response to ALD. These results indicate that γδ T cells are dispensable for NBP-induced peritonitis in mice. In addition, pretreatment of mice with i.p. administration of lovastatin (2.46 μmol per mouse on days −1, 0, 1, and 2) to prevent ALD-induced accumulation of isopentenyl-5-pyrophosphate (19) did not inhibit the ALD-induced leukocyte infiltration (leukocyte number \times 10^6 ± SEM: ALD – 22.6 ± 6.3, lovastatin + ALD – 25.2 ± 2.1; n = 4, p = 0.7), indicating further that the pyrophosphate metabolites activating γδ T cells (5) are not exclusively responsible for ALD-induced peritoneal inflammation. Pretty2 and W/Wv mice are deficient in melanocytes and mast cells owing to mutations in the c-kit gene (12). ALD did not cause a significant influx of cells into the peritoneum of the two mast cell-deficient mouse strains (Fig. 3A, 3B). Moreover, reconstitution of W/Wv mice with WT mast cells restored their ALD responsiveness (Fig. 3B). These results confirm a role for mast cells in ALD-induced peritonitis. Mast cell degranulation was not required for the inflammatory actions of ALD, because inhibition of degranulation with cromolyn, or exhaustive prior degranulation with compound...
IL-1 is necessary for NPB-induced inflammation

NBP-induced total leukocyte and neutrophil infiltration into the peritoneal cavity were both dependent on MyD88 signaling, whereas IL-1R deficiency significantly diminished only the infiltration of neutrophils (Fig. 4A, 4B). IL-1α and IL-1β deficiencies similarly decreased the numbers of neutrophils in ALD-induced peritonitis (Fig. 4B). Mice deficient in factors deficiencies similarly decreased the numbers of neutrophils in NBP-induced total leukocyte and neutrophil infiltration into the peritoneal cavity were both dependent on MyD88 signaling. IL-1α is necessary for NPB-induced inflammation with a model in which mast cells are a pre-existing source of IL-1, and IL-1β did not attenuate the proinflammatory effects of NBP (Fig. 4C).

Cocultivation of mast cells with peritoneal mesothelial cells induced an increase in IL-6 production (Fig. 4D, 4E). Furthermore, when ZLD, an NBP more potent than ALD, was added to the mast cell-mesothelial cell cocultures, additional IL-6 was released, which required IL-1R expression by the mesothelial cells (Fig. 4D). Treatment of isolated mast cells with ZLD caused a selective release of IL-1α, compared with IL-6 (Fig. 4E), suggesting that NPBs stimulated mast cells to release IL-1, which in turn activated the IL-1R on the mesothelial cells.

In summary, NBP-induced peritoneal inflammation depends upon mast cells and IL-1R signaling for neutrophil recruitment. The NPBs are rapidly cleared from plasma and sequestered in bone (22). The t1/2 of ALD in bone is $\geq 200$ d in rodents and $>1000$ d in dogs (22). Hence, ALD and other NPB concentrations in bone progressively rise with time and can attain high local levels. Thus the pharmacokinetics of NPBs, and their ability to recruit neutrophils, as demonstrated in this article, may explain the inflammation and osteonecrosis that can occur in humans after chronic administration.

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Disclosures

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