Inhibitory Effect of KBT-3022, a New Anti-platelet Agent, on Infiltration of Polymorphonuclear Leukocytes Induced by Leukotriene B$_4$ or Formyl-Methionyl-Leucyl-Phenylalanine in Mice

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ABSTRACT—We devised a method for evaluating polymorphonuclear leukocyte (PMN) infiltration in vivo employing an air bleb technique combined with measurement of myeloperoxidase (MPO) activity, and the effects of some anti-platelet agents were evaluated. KBT-3022 (ethyl 2-[4,5-bis(4-methoxyphenyl)thiazol-2-yl]pyrrol-1-ylacetate) and cilostazol inhibited the increase in MPO activity in the connective tissue around the air bleb induced by leukotriene B$_4$ (LTB$_4$) and formyl-methionyl-leucyl-phenylalanine (fMLP). Indomethacin inhibited only the fMLP-induced increase in MPO activity, but ticlopidine hydrochloride and acetylsalicylic acid had no effect. Histologic observation confirmed the inhibition of PMN infiltration by KBT-3022. These results indicate that KBT-3022 may be a potent inhibitor of both LTB$_4$- and fMLP-induced infiltration of PMNs.

Keywords: Leukotriene B$_4$, Formyl-methionyl-leucyl-phenylalanine, KBT-3022

The polymorphonuclear leukocyte (PMN) plays an important role not only in host defense but also in aggravation of tissue injury during ischemia and after reperfusion (1). Therefore, in these pathological conditions, suppression of PMN activation seems to be therapeutically advantageous.

PMNs are known to be activated by the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP), a synthetic oligopeptide resembling the chemotactic factors produced by bacteria (2), and leukotriene B$_4$ (LTB$_4$) (3). Furthermore, some chemotactic agents have been reported to activate PMNs (4, 5). Simple and reproducible methods are needed to search for and evaluate non-specific or agonist-specific inhibitors of PMN activation in vivo.

The air bleb technique was originally developed in rats by Higginbotham (6) for studying mast cell degranulation, and it was later modified by Clark et al. (7), Lawman et al. (8) and others. They used a histologic technique for quantifying PMN accumulation, but this was time-consuming because it required large numbers of observations on multiple tissue sections. We have established a simple method for evaluating PMN infiltration through vessels by measuring PMN-specific myeloperoxidase (MPO) activity in mice. Moreover, using this method, the in vivo effects of ethyl 2-[4,5-bis(4-methoxyphenyl)thiazol-2-yl]pyrrol-1-ylacetate (KBT-3022) (9), a new anti-platelet agent, on LTB$_4$- and fMLP-induced PMN infiltration were investigated in comparison with those of acetylsalicylic acid (ASA), ticlopidine hydrochloride (TP), cilostazol, indomethacin and dexamethasone.

Blood was taken from the inferior vena cava of mice (weighing 21–29 g, male ddY; Japan SLC, Hamamatsu) under ether anesthesia into syringes containing 0.1 ml of 3.8% trisodium citrate per 0.9 ml of blood. Blood samples from mice were pooled and mixed with 3% dextran-saline (M.W.: 208,000; Nacalai Tesque, Kyoto) and left to stand at 4°C for 40 min to sediment the erythrocytes. Cells in the supernatant were pelleted by centrifugation and resuspended in 1 ml of phosphate-buffered saline, pH 7.4 (PBS). This suspension was layered on 5 ml of Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada) and centrifuged at 500 × g for 20 min at 25°C. Erythrocytes remaining in the cell pellets were lysed with cold water for 30 sec. The resulting cells were washed twice and resuspended in PBS. The cell suspension was more than 90% neutrophils, as assessed by May-Grünwald-Giemsa-stained smears, and had a viability of
more than 99% as assessed by Trypan Blue exclusion. The neutrophil preparations were stored at −70°C until the assay for MPO activity.

The infiltration of PMNs into the subcutaneous tissue in mice was estimated by the method described by Lawman et al. (8) with some modifications. Briefly, an air bleb was formed by injection of 1 ml of air via a 27G needle into the subcutaneous connective tissue on the back of the mice. Immediately, 0.3 ml of 1 μM LTB₄ (Cayman Chemical, Ann Arbor, MI, USA) was injected into the air bleb. In sham-treated animals, saline alone was injected. Mice were given food and water ad libitum before and during the experiments. At 2 hr after the injection of the chemoattractant, the mice were sacrificed with ether, and the skin surrounding the air bleb was surgically excised. A portion of the dorsal surface of the thin connective tissue around the air bleb was placed in contact with a glass slide on which a small circle of binding agent had been laid. After drying, the thin connective tissue fixed to the glass slide was removed. An area of approximately 50 mm² of connective tissue (wet weight: approximately 1 mg) was cut off and stored at −70°C until the assay for MPO activity. Alternatively, the connective tissue was stained with May-Grünewald-Giemsa solution for morphological determination. The morphology of 1000 infiltrating leukocytes was observed under a light microscope at a magnification of ×400. The percentages of neutrophils, eosinophils and monocytes were determined.

KBT-3022 was synthesized at Kanebo, Osaka. TP and cilostazol were extracted and purified from Panaldine® (Daichi Pharmaceutical, Tokyo) and Pletaal® (Otsuka Pharmaceutical, Tokyo), respectively, at Kanebo. ASA and dexamethasone from Wako Pure Chemical Industries, Osaka and indomethacin from Sigma were used. All agents were dissolved or suspended in 0.5% polyoxyethylene sorbitan monooate solution (Wako Pure Chemical Industries). Dexamethasone was administered orally 3 hr before and the other drugs were administered orally 1 hr before chemoattractant injection.

The MPO activity was measured according to the method described by Bradley et al. (10) with some modifications. Briefly, the connective tissue around the air bleb or neutrophils prepared from peripheral blood were suspended in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide (Sigma) and sonicated in an ice bath for 60 sec using an Ultrasonic Processor (Heat Systems-Ultrasonics; Farmingdale, NY, USA). After centrifugation at 40,000×g for 15 min at 4°C, 0.1 ml of the supernatant was mixed with 0.9 ml of 50 mM potassium phosphate buffer (pH 6.0) containing 0.167 mg/ml o-dianisidine dihydrochloride (Sigma) and 0.0005% hydrogen peroxide. After incubation for 3 hr at 25°C, the absorbance at 460 nm was measured in a spectrophotometer.

The relationship between the number of neutrophils (2.5×10⁴–2×10⁵ cells) and MPO activity was linear, indicating that the potency of the MPO activity was accurately reflected by the amount of neutrophils (OD: 1.37×cell number / 10⁶–0.0254, r=0.999). In humans, the MPO content per neutrophil is more than 5% dry weight, and monocytes have a low content of MPO (11).

Preliminary results demonstrated that LTB₄ at 0.1–3 μM caused a concentration-dependent increase in the MPO activity in the connective tissue around the air bleb 2 hr after LTB₄ injection. The sub-maximum increase in MPO activity was obtained at 1 μM LTB₄ and reached a plateau at more than 3 μM LTB₄ (data not shown). Similar results were obtained for the fMLP-induced increase in MPO activity, and the sub-maximum increase was obtained at 3 μM fMLP (data not shown).

Injection of 1 μM LTB₄ or 3 μM fMLP into the air blebs produced marked increases in MPO activity in the connective tissue around the air bleb in control animals (Figs. 1 and 2). KBT-3022 at 1–10 mg/kg, p.o. inhibited the LTB₄-induced increase in MPO activity significantly. Cilostazol at 10 mg/kg, p.o. and dexamethasone at 1 mg/kg, p.o. also inhibited the LTB₄-induced increase in MPO activity significantly, but ASA, TP and indomethacin did not have any effect (Fig. 1). Similarly, KBT-3022 at 0.3–3 mg/kg, p.o. inhibited the fMLP-induced increase in MPO activity significantly. Cilostazol at 10 mg/kg, p.o., indomethacin at 10 and 30 mg/kg, p.o. and dexamethasone at 1 mg/kg, p.o. also inhibited the fMLP-induced increases in MPO activity significantly, but ASA and TP did not show any effect (Fig. 2). All the drugs tested including KBT-3022 and its metabolite desethyl KBT-3022 had no effect on the activity of MPO itself in vitro (data not shown).

Histologic observation was performed to confirm the morphology of the infiltrating cells and the inhibitory effects of KBT-3022 on leukocyte infiltration. Figure 3 shows a typical preparation of connective tissue around an air bleb 2 hr after injection of chemoattractant. Marked infiltration of PMNs into the connective tissue was observed in control animals. In the absence of a chemoattractant, few leukocytes infiltrated. The leukocytes infiltrated in response to fMLP comprised 99.10% neutrophils, 0.5% eosinophils and 0.4% monocytes. On the other hand, the leukocytes infiltrated in response to LTB₄ comprised 87.4% neutrophils, 12.3% eosinophils and 0.3% monocytes. Oral administration of KBT-3022 markedly inhibited neutrophil infiltration primarily induced by LTB₄ and fMLP at 3 and 1 mg/kg, respectively (Fig. 3, C and F). The infiltration of eosinophils induced by LTB₄ also seemed to be inhibited after oral administra-
tion of KBT-3022 because the percentage of eosinophils among the infiltrating leukocytes was almost unchanged. The eosinophil is reported to contain approximately 3 times more potent peroxidase activity than the neutrophil (12), and so LTB4 may induce a higher MPO activity than fMLP. A similar phenomenon was also observed in preparations obtained 4 hr after chemoattractant injection (data not shown).

Oral administration of KBT-3022, cilostazol and dexamethasone inhibited both the LTB4- and fMLP-induced increases in MPO activity in the connective tissue around the air blebs. Indomethacin inhibited only the fMLP-induced increase in MPO activity. Cilostazol is one of several potent cAMP-phosphodiesterase inhibitors that are reported to suppress cellular responses by inhibiting the mobilization and/or influx of calcium ions (13). Dexamethasone has been reported to inhibit the neutrophil extravasation induced by several agonists (14). On the other hand, indomethacin was a specific inhibitor of fMLP-induced PMN stimulation (15). KBT-3022 and its metabolite inhibit LTB4 and fMLP-induced migration and increases in the intracellular free calcium concentration in vitro, although its inhibitory mechanisms have not yet been elucidated (K. Yokota, unpublished data). These results suggest that our air bleb technique combined with measurement of MPO activity is useful for the evaluation of inhibitors of PMN infiltration in vivo.

In summary, using mice, an experimental model for evaluating LTB4- and fMLP-induced PMN infiltration was established, and the effect of KBT-3022 was investigated. Oral administration of KBT-3022 inhibited both the LTB4- and fMLP-induced increases in MPO activity in the connective tissue. These results indicate that KBT-3022 may be a potent inhibitor of both LTB4- and fMLP-induced infiltration of PMNs in vivo.
Fig. 3. Representative photomicrographs. Effect of KBT-3022 on LTB₄ (A–C)- and fMLP (D–F)-induced PMN infiltration in mice. May-Grunwald-Giemsa-stained preparations of the connective tissue around the air bleb 2 hr after LTB₄ or fMLP injection. A: control (LTB₄ injection), B: sham (saline instead of LTB₄), C: KBT-3022 (3 mg/kg, p.o. at 1 hr before LTB₄ injection), D: control (fMLP injection), E: sham (saline instead of fMLP), F: KBT-3022 (1 mg/kg, p.o. at 1 hr before fMLP injection). The bar represents 50 μm.
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