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INHIBITION BY THE LTD_4 ANTAGONIST, SR2640, OF EFFECTS OF LTD_4 ON CANINE POLYMORPHONUCLEAR LEUKOCYTE FUNCTIONS

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Abstract—The sulfidopeptide leukotriene LTD_4 selectively inhibited directed migration of canine polymorphonuclear leukocytes towards LTB_4 (100 nM) with an IC_{50} of 38 nM. No effect on PAF-acether induced migration was observed. LTD_4 did not cause detectable adherence of cells to the Boyden chamber system. Neither LTD_4 nor the LTD_4/LTE_4 receptor antagonist, SR2640, possessed chemokinetic properties. LTD_4 induced reversible aggregation of the cells with an EC_{50} of 36 nM. SR2640 suppressed or abolished LTD_4 responses *in vitro* and following oral administration of the drug. SR2640 had no effect on aggregation induced by LTB_4 or PAF-acether. The results can be taken as evidence that canine polymorphonuclear leukocytes possess LTD_4 receptors that may be involved in a leukotriene specific micro-feedback system between the different cell types involved in inflammatory responses.

A variety of proinflammatory mediators are capable of inducing some or all of the polymorphonuclear leukocyte (PMN) functions: aggregation, adhesion, chemotaxis, secretion of proteases, phagocytosis and the respiratory burst [1]. Leukotriene (LT) B_4 is such a mediator [2, 3] and consequently, LTB_4 has been ascribed a role in the development and maintenance of inflammation [4].

In contrast, the cysteinyl-containing sulfidopeptide leukotriene constituents of slow-reacting substance of anaphylaxis (SRS-A), LTC_4, LTD_4 and LTE_4 [5], show few effects on human PMN functions. These leukotrienes are primarily known as spasmogenic agents affecting tracheo-bronchial and intestinal smooth muscle in guinea-pig and man, among other species [6–8]. They also enhance mucus secretion [9] and possess vasoactive properties [10]. Therefore, sulfidopeptide leukotrienes have, in particular, been implicated as mediators of obstructive airway disease and a number of sulfidopeptide leukotriene receptor antagonists have been developed [11–14], and are now undergoing clinical trials in bronchial asthma.

We have recently described LTD_4 receptors and their intracellular signal transduction mechanism in human PMN [15]. The novel, specific LTD_4/LTE_4 receptor antagonist, SR2640 [11], was used in the study to characterize the LTD_4 receptors, and the effects of LTD_4 and SR2640 on a single PMN function, chemotaxis, were also reported [15]. The aim of the present investigations was to study the effects of LTD_4 on canine PMN chemotaxis and aggregation *in vitro* and the modulation of these effects by SR2640 *in vitro* and after oral administration of the drug.

MATERIALS AND METHODS

Chemicals. SR2640 (S. Rachlin, Leo Pharmaceutical Products, Denmark) was used as the potassium salt, dissolved in 0.1 N NaOH and diluted to the desired concentrations in 0.5 N Tris–HCl buffer, pH 7.4. For *in vivo* experiments, SR2640 was administered as 250 mg tablets. Stock solutions of 10 mM LTB_4 and LTD_4 (Ultrafine Chemicals, U.K.) in ethanol were diluted in Hank's balanced salt solution (HBBS; Gibco, U.S.A.) immediately prior to use. Platelet activating factor (PAF-acether) (Sigma Chemical Co., St. Louis, MO, U.S.A.) was diluted in HBSS containing 0.2% bovine serum albumin (BSA) (Sigma Chemical Co.). Methylcellulose (Methocel 15, Dow Chemicals, U.S.A.), and Lymphoprep (Nycomed, Norway) were used for purification of PMN, and MicroWell Modules (Nunc, Denmark) separated by 3 μm pore-sized nitrocellulose filters (Sartorius, F.R.G.) constituted the chemotaxis chambers.

Isolation of PMN. Twenty to 80 ml EDTA-stabilized blood was collected from the cephalic vein of healthy Beagle dogs of either sex, weighing 12–13 kg, that had not received any drugs the last two weeks before an experiment. For oral dosing experiments, blood was collected immediately prior to and 1.5 hr after administration of SR2640. An untreated control dog was included in all experiments. All the following steps were carried out at 20°. Leukocyte-rich plasma, obtained by sedimentation for 45 min in 0.8% methylcellulose in non-adsorbent tubes, was gently layered on 4 ml Lymphoprep and centrifuged at 400 *g* for 30 min. In aggregation experiments, erythrocytes were lysed for 30 sec in 0.2% saline, isotonicity was reestablished, and the cells were centrifuged, resuspended and washed at 200 *g* for 10 min in divalent cation-free HBSS. In chemotaxis experiments, cells were washed twice in HBSS enriched with 2% BSA and 0.2% glucose (enriched HBSS) at 200 *g* for 10 min. Cell density was adjusted to 2×10^6/ml or $3–5 \times 10^7$/ml for chemotaxis and aggregation experiments, respectively. The final cell suspensions contained more than 95% PMN with a recovery of approximately 45%. Cell viability was 98% as determined by the Eosin Y exclusion test.

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Chemotaxis. A modified Boyden chamber [16] consisting of MicroWell Modules was used and contained an upper cell compartment and a lower chemoattractant compartment. To the former was added 400 μl PMN suspended in enriched HBSS and preincubated with or without 100 nM SR2640 for 5 min at 37° followed by a similar preincubation with 0–100 nM LTD_4. Random migration (RM) was estimated by measuring migration towards enriched HBSS after an incubation period of 45 min at 37°. Chemotaxis was assayed towards the optimal concentration, 100 nM, of LTB_4 or PAF-acether. Immediately after incubation, filters were fixed and stained with haematoxylin [17]. Migration was estimated 48 hr later according to the leading front technique [18] by recording the mean of two median values obtained after examination of five fields on duplicate filters. The effect of LTD_4 or SR2640 plus LTD_4 (sample) on normal LTB_4 or PAF-acether directed migration (control) was calculated as follows:

% inhibition of chemotaxis = [1−(sample−RM)/(control−RM)] × 100%.

In order to evaluate the possibility of LTD_4-induced adherence of PMN to the chamber system, a cell count was performed on the cell suspension after 45 min incubation.

Aggregation. Two hundred μl PMN suspension, 5 μl recalcification medium (HBSS containing 40 mM $CaCl_2$ and 30 mM $MgCl_2$) and a siliconized magnetic stirrer were placed in a siliconized aggregometer cuvette. The light transmission with and without the cuvette in the light path was set to 10 and 90%, respectively. When the baseline was stable, 5 μl of SR2640 or HBSS was added and one minute later, 5 μl of LTD_4 or LTB_4 in 4% ethanol was added. The aggregation curve was subsequently followed for 5 min in a Payton aggregometer set at 37° and 900 rpm. The maximum increase in percentage light transmission (ΔT) was measured, and inhibition of aggregation was calculated as follows:

% inhibition of LTD_4-induced aggregation = (1−ΔT(SR2640 present)/ΔT(SR2640 absent)) × 100%.

Aggregation and chemotaxis experiments were also performed using PMN from blood samples obtained after oral administration of SR2640 to the dogs.

Statistics. Student's paired *t*-test was used for analysis of all data. Results are presented as mean values ± SE.

RESULTS

PMN migrated in a concentration-related manner towards LTB_4 and PAF-acether (Fig. 1) and 100 nM of either chemoattractant was chosen for further experiments.

PMN that were incubated in the presence of 1–100 nM LTD_4 exhibited a dose-dependent and significant suppression of LTB_4-directed migration (Fig. 2). The IC_{50} for inhibition of chemotaxis by LTD_4 was 38 nM. The maximum inhibition was 65%. When the cells were preincubated with 100 nM SR2640 prior to addition of LTD_4, the inhibition of migration was abolished (Fig. 2). With PAF-acether as chemoattractant, LTD_4 only caused marginal and non-significant inhibition of directed migration (Fig. 3). To evaluate possible chemokinetic properties of LTD_4 and SR2640, migration was assayed with 100 nM LTD_4 or SR2640 in the cell compartment and enriched HBSS in the chemoattractant compartment. Migration under these conditions was 46 ± 7 and 44 ± 6 μm/hr (N = 6), respectively, thus not differing significantly from the normal random migration (44 ± 4 μm/hr, N = 6). In order to discriminate between true migration inhibition and

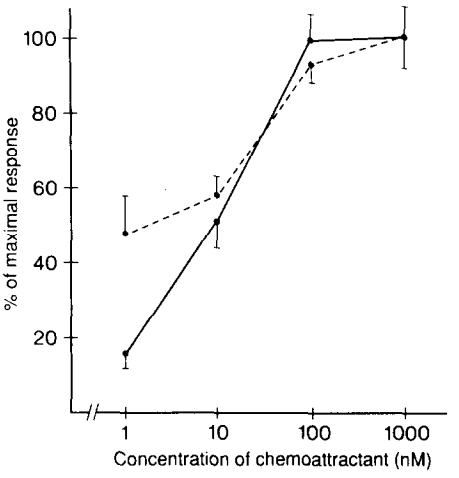

Fig. 1. Migration of canine PMN towards LTB_4 (full line) and PAF-acether (dotted line). Values represent the percentage of the chemotactic differential obtained at 1000 nM. Results shown are means ± SE of 4 (LTB_4) and 3 (PAF-acether) experiments.

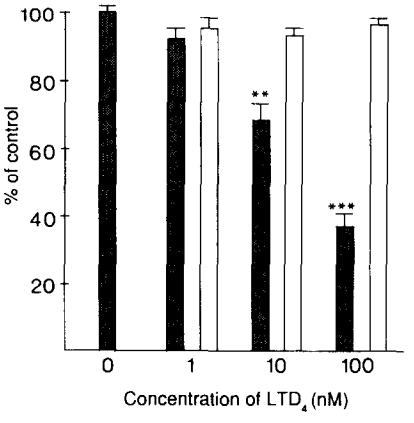

Fig. 2. Inhibition by LTD_4 of LTB_4-directed migration of canine PMN and abolishment of inhibition by SR2640. Cells were pretreated with or without 100 nM SR2640 followed by 0–100 nM LTD_4 and migration towards 100 nM LTB_4 was assayed. Each column and bar represents the mean ± SE of 12–13 (closed columns: SR2640 absent) or 7–8 (open columns: SR2640 present) experiments. $^{**}P < 0.01$; $^{***}P < 0.001$.

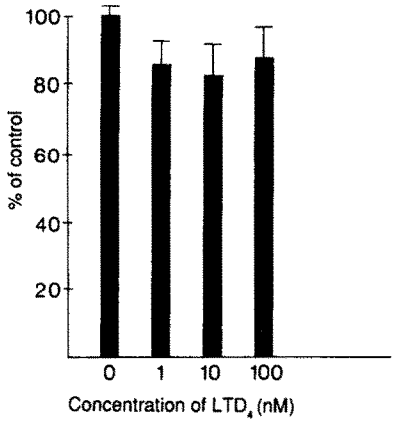

Fig. 3. Effect of LTD_4 on PMN migration induced by PAF-acether. Cells were preincubated with 0–100 nM LTD_4 and migration towards 100 nM PAF-acether was determined. Each column and bar represents the mean ± SE of 3 experiments.

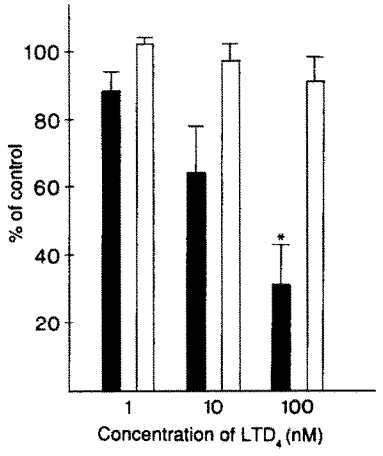

Fig. 4. Abolishment of LTD_4-induced migration inhibition by oral administration of SR2640. Cells obtained immediately before (closed columns) administration of 500 mg SR2640 to 3 dogs, and 1.5 hr after (open columns) drug administration, were preincubated with 0–100 nM LTD_4, and migration towards 100 nM LTB_4 was measured. *$P < 0.05$.

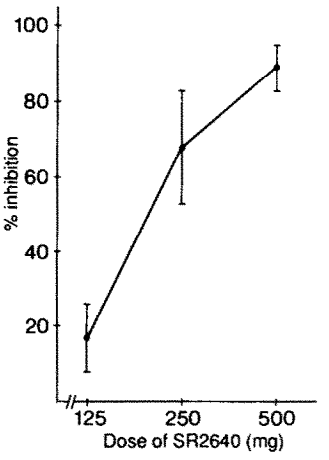

Fig. 5. Dose–response relationship for inhibition of LTD_4-induced migration inhibition following oral administration of SR2640. Cells obtained before and 1.5 hr after administration of SR2640 were preincubated with 100 nM LTD_4, and migration towards 100 nM LTB_4 was measured. The percentage inhibition of the LTD_4-effect at 0 hr is shown. Means ± SE of 5–7 experiments are shown.

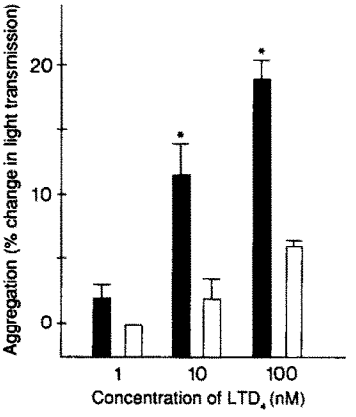

Fig. 6. Aggregation of PMN induced by LTD_4 and inhibition of aggregation by SR2640. Cells were briefly preincubated with (open columns) or without (closed columns) 100 nM SR2640, and the aggregatory response elicited by 1–100 nM LTD_4 was measured. Each column and bar represents the mean ± SE of 3 experiments. *$P < 0.05$.

chamber adherence phenomena, PMN were incubated for 45 min with and without 100 nM LTD_4, and a cell count was performed on the chamber fluid succeedingly. Non-adherent cell numbers were determined to be $1.50 \pm 0.18 \times 10^6$ in the absence ($N = 4$), and $1.55 \pm 0.25 \times 10^6$ in the presence ($N = 4$) of LTD_4, respectively. This indicates that the number of non-adherent cells did not decrease significantly during incubation with LTD_4.

Cells were isolated from dogs immediately prior to, and 1.5 hr after, administration of 500 mg SR2640 and a concentration–response relationship for inhibition by LTD_4 of LTB_4-induced migration was established at both times. PMN isolated after treatment with 500 mg SR2640 were virtually unresponsive to inhibition by LTD_4 (Fig. 4). Oral experiments were also carried out with 125 and 250 mg SR2640 and a dose–response relationship for inhibition of the LTD_4-effect was found (Fig. 5). ED_{50} was calculated to be 195 mg SR2640, corresponding to approximately 17 mg/kg.

In aggregation experiments, addition of LTD_4 at increasing concentrations caused transient and concentration-related aggregation of PMN with a maximum increase in light transmission (ΔT) of 33% at 1 μM LTD_4 and an EC_{50} of 36 nM (Fig. 6). Aggregation was maximal within 30 sec and had been completely reversed after 2 min (Fig. 7). The LTD_4/LTB_4 potency ratio was approximately 1:4. Cells preincubated for 1 min with 100 nM SR2640 showed a diminished aggregatory response to LTD_4 (Fig.

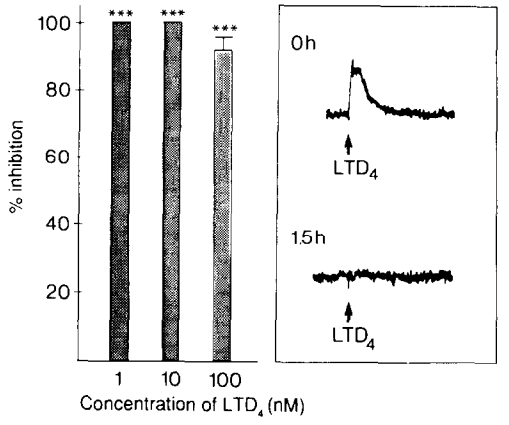

Fig. 7. Inhibition of LTD_4-induced PMN aggregation following oral administration of SR2640. The aggregation of PMN induced by 1–100 nM LTD_4 was measured immediately prior to and 1.5 hr after, oral administration of 500 mg SR2640 to 3 dogs. The insert shows the response to 10 nM LTD_4 before and after SR2640 administration.

6). SR2640 had no effect on LTB_4 or PAF-acether induced aggregation (data not shown).

For studies of PMN aggregation after oral administration of SR2640, cells were obtained immediately before, and 1.5 hr after administration of 500 mg SR2640. The LTD_4-induced aggregation observed at 0 hr was almost completely abolished at 1.5 hr, with only slight aggregation being present at 100 nM LTD_4 (Fig. 7).

DISCUSSION

Stimulation of human PMN with LTD_4 has recently been described [15] and was shown to involve activation of the PI response (increase in intracellular levels of inositol phosphates and free Ca^{2+}). The present experiments which investigated the functional responses of canine PMN to LTD_4, strongly indicate that PMN in this species also possess dynamic LTD_4 receptors. Based on EC_{50} and IC_{50} values of 36–38 nM for aggregation and inhibition of chemotaxis, respectively, the presence of high affinity LTD_4 receptors on circulating canine PMN may be postulated. This is substantiated by the capability of the novel LTD_4/LTE_4 receptor antagonist, SR2640, to suppress or abolish the functional responses. These effects were obtained at 100 nM SR2640. At this concentration, SR2640 is a potent LTD_4 receptor antagonist, but shows no effect on arachidonic acid metabolism [11]. The prevention of endogenous prostaglandin or leukotriene synthesis is thus not a likely explanation of the effects observed with SR2640. The results indicate that structural requirements for an antagonist to act on the PMN receptor apparently are similar to those for binding to airway and intestinal LTD_4 receptors, since these were utilized to characterize the pharmacological properties of SR2640 [11]. This suggests homogeneity of LTD_4 receptors in various cell types and animal species. One species discrepancy between human and canine PMN is the apparent two-fold greater inhibition of LTB_4-directed migration by LTD_4 attainable in canine PMN. In human PMN, maximum inhibition was approximately 35% [15].

The experiments with oral administration of SR2640 indicated that the drug is not readily displaced from PMN receptors during cell preparation. An explanation for this is that either a strong receptor–ligand interaction is present on these cells or the PMN contain mobilizable, intracellular organelle-associated receptor sites. The latter would be in accordance with recent results showing that the majority of human PMN receptors for another sulfidopeptide leukotriene, LTC_4, are sited on lysosomal granules [19]. In this case, the described procedure would be applicable for determination of the efficacy of other oral leukotriene antagonists.

Human alveolar macrophages contain high affinity LTD_4 receptors [20]. Furthermore, LTC_4 and LTD_4 cause adherence of PMN to microvascular endothelium [21] and synthetic surfaces [22] and selectively inhibit migration of human PMN towards LTB_4 [23]. Thus, evidence indicating modulation of the cellular phase of inflammation by sulfidopeptide leukotrienes is increasing. Sulfidopeptide leukotriences are among the primary products synthesized by macrophages [24], eosinophils [25] and mast cells [26], in contrast to PMN [25, 27]. A tempting, albeit speculative hypothesis is that a leukotriene specific micro-feedback system exists between the former cell types on the one hand and PMN on the other. Thus, sulfidopeptide leukotrienes synthesized by the first-mentioned cell types could act on PMN, attracted to the area by an LTB_4-gradient (for a review, see [4]), to immobilize the cells in, and thus prevent emigration from the inflamed area in a manner somewhat analogous to that seen with the lymphokine, leukocyte migration inhibition factor (LIF) [28, 29]. This would imply that sulfidopeptide leukotrienes do not deactivate PMN, as the present results otherwise might suggest, but rather are involved in maintenance of PMN infiltration in inflamed tissue.

If LTD_4 modulates the cellular phase of inflammatory reactions, then LTD_4 receptor antagonists, developed as smooth muscle relaxants, may have a potential as antiinflammatory drugs in certain conditions.

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