Transcellular metabolism of leukotriene A_4 by rabbit blood cells: lack of relevant LTC_4-synthase activity in rabbit platelets

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Abstract The objective of this study was to determine the transcellular metabolism of leukotriene A_4 by rabbit blood cells. Mixed peripheral blood leukocyte preparations with and without platelets in a ratio of 1:40 were challenged with the Ca^{2+}-ionophore A23187. 5-Lipoxygenase metabolites production was assessed by RP-HPLC coupled with diode-array UV detection. In light of the observation that leukotriene C_4 production in leukocyte–platelet coincubation was the same as with leukocytes alone, mixed coincubation of human and rabbit blood cells was tested. Rabbit leukocytes with human platelets resulted in a significant increase of leukotriene C_4 production, while no changes were observed in human leukocytes with or without rabbit platelets. In agreement with these results, intact rabbit platelets or rabbit platelet lysates, unlike human platelets, were not able to convert synthetic leukotriene A_4 free acid to leukotriene C_4. These data provide evidence that rabbit leukocytes are able to make a significant amount of leukotriene A_4 available for transcellular metabolism, while rabbit platelets, unlike human platelets, lack leukotriene C_4-synthase activity.—**Sala, A., T. Testa, F. Nobili, and G. Folco.** Transcellular metabolism of leukotriene A_4 by rabbit blood cells: lack of relevant LTC_4-synthase activity in rabbit platelets. *J. Lipid Res.* 1997. **38**: 627–633.

Supplementary key words human platelets • rabbit leukocytes

Leukotrienes (LT), e.g., leukotriene B_4 (LTB_4) and leukotriene C_4 (LTC_4), are products of the 5-lipoxygenase (5-LO; EC 1.13.11.34) pathway of arachidonic acid (AA) metabolism and are potent biologically active autacoids. They are involved in microvascular inflammatory responses, where LTC_4 and LTD_4 are able to affect vascular permeability, causing plasma extravasation, and LTB_4 has specific effects on the adhesion of neutrophils to endothelial cells and extravasation of white cells (1). The generation of LT exhibits remarkable cellular specificity; polymorphonuclear leukocytes (PMNL) generate predominantly the dihydroxy-derivative LTB_4, with only minor amounts of LTC_4, whereas mast cells and eosinophils show preferential generation of cysteinyl-leukotrienes (cys-LT) (2). Recently another process of biosynthesis of cys-LT has been described, where the unstable metabolic intermediate LTA_4 is further metabolized by vicinal cells (possessing the LTC_4-synthase but not the 5-lipoxygenase enzyme) into leukotriene C_4. Such a reaction involves the cooperation of PMNL with platelets, endothelial cells, and smooth muscle cells (3–5). This process has been termed "transcellular biosynthesis" and suggests that the cellular environment (i.e., cell–cell interaction) exerts an important control on the production of eicosanoids (6).

Recent studies on transcellular metabolism in complex organ systems (7–10) showed that perfusion and activation of PMNL in isolated lung or heart of the rabbit resulted in the production of significantly increased amounts of cysteinyl leukotrienes, compared to activation of PMNL alone. These biochemical changes were associated with significant functional and morphological changes, suggesting that transcellular biosynthesis of cysteinyl leukotrienes might indeed be of physiopathological relevance. The results are of interest with respect to the potential of rabbit blood cells in the transfer and metabolism of leukotriene A_4.

We have studied the transcellular metabolism of leukotriene A_4 using rabbit mixed leukocyte preparations and coincubations of leukocytes and platelets. The abil-

Abbreviations: LTA_4, leukotriene A_4; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; PMNL, polymorphonuclear leukocytes; 5-LO, 5-lipoxygenase; PBS^-, phosphate-buffered saline without Ca^{2+} and Mg^{2+}; PRP, platelet-rich plasma; 5,12-diHETE, 5(S),12(S)-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid; CDNB, 1-chloro-2,4-dinitrobenzene; RP-HPLC, reverse phase high performance liquid chromatography; AA, arachidonic acid.

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ity of rabbit leukocytes to transfer leukotriene A_4 and of rabbit platelets to synthesize LTC_4, has been tested using heterologous coincubations with, respectively, human platelets and leukocytes.

The results showed that rabbit platelets do not synthesize LTC_4 when coincubated with leukocytes or when exposed to synthetic LTA_4 free acid. On the other hand, rabbit leukocytes are able to make significant amounts of intact LTA_4 available for transcellular metabolism.

EXPERIMENTAL PROCEDURES

Chemicals and reagents

All chemicals used were reagent grade and obtained from commercial sources. Eicosanoids were purchased from Cayman Chemical Co. (Ann Arbor, MI). HPLC-grade solvents were obtained from Merck (Darmstadt, Germany). Type I "plus" water was obtained using a MilliQ Plus water purifier (Millipore, Molsheim, France) with double distilled water.

Preparations of rabbit and human blood cells

After centrifugation for 20 min at room temperature and 200 *g*, platelet-rich plasma (PRP) was removed, re-acidified with ACD (1/10 of the volume) and centrifuged for 15 min at room temperature and 1000 *g*. Pelleted platelets were resuspended with 5 ml of washing buffer (36 mM citric acid, 5 mM glucose, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2{\cdot}6H_2O$, 103 mM NaCl, pH 6.5) containing 0.4%, w/v, BSA and prostaglandin E_1 (final concentration 100 nM). Platelets were further centrifuged for 15 min at room temperature and 800 *g* and finally resuspended in phosphate-buffered saline without Ca^{2+} and Mg^{2+} ($PBS^{=}$).

Leukocytes were prepared from residual blood by dextran sedimentation and hypotonic lysis of contaminating red cells. Mixed leukocytes were washed twice with $PBS^{=}$ and resuspended in $PBS^{=}$. Viability (≥95%) was assessed by exclusion of Trypan Blue dye.

Rabbit polymorphonuclear leukocytes were purified from mixed leukocytes by centrifugation on Ficoll cushions (d 1.077 g/ml) for 30 min at room temperature and 400 *g*.

Human blood (40 ml) was drawn from healthy donors that had not taken any medications for at least 1 week; it was collected into a 50-ml polypropylene centrifuge tube containing 5.7 ml of ACD and carefully mixed. After centrifugation for 15 min at room temperature and 280 *g*, platelet-rich plasma (PRP) was removed; platelets and mixed leukocytes were prepared as described for rabbit cells.

Platelets and leukocytes were counted using a modified Neubauer chamber.

Cell incubations

Rabbit or human leukocytes (5×10^6 cells ml^{-1}) and/or homologous or heterologous platelets (2×10^8 cells ml^{-1}) were treated with Ca^{2+} (2 mM) and Mg^{2+} (0.5 mM) and, after pre-incubation at 37°C for 5 min, the calcium ionophore A23187 (2 μm) (Calbiochem, La Jolla, CA) was added to trigger eicosanoid metabolism.

Stimulation was terminated after 10 min with 2 vol of ice-cold methanol containing the HPLC internal standard PGB_2 (25 ng), and samples were stored at −20°C until RP-HPLC analysis.

Metabolism of synthetic LTA_4 by intact platelets

LTA_4 free acid was obtained through basic hydrolysis of LTA_4 methyl esther (LTA_4-ME). Briefly, LTA_4-ME was reconstituted in ice-cold acetone–0.25 M NaOH 4:1 (v/v) and hydrolysis was performed at room temperature for 60 min. LTA_4 free acid was added to rabbit (10^8–4×10^8 cells) or human platelets (10^8 cells) containing BSA (0.5%, w/v) within 1 h of hydrolysis, at different final concentrations (0.2–2 μM). Purity of LTA_4-ME was checked by normal phase HPLC, using cyclohexane–ethyl acetate–triethylamine 99:0.5:1 (v/v) to isocratically elute a Lichrospher Si-100 column (4 × 250 mm, 5 μm; Merck) at a flow rate of 1 ml/min. LTA_4 free acid was checked by reverse phase HPLC using acetonitrile–0.01 M borate buffer, pH 10, 40:60 (v/v) to isocratically elute an Ultrasphere RP-18 column (4 × 250 mm, 5 μm; Beckman Analytical, Palo Alto, CA) at a flow rate of 1 ml/min (11).

UV absorbance was monitored at 280 nm and full UV spectra (240–340 nm) were acquired at a scan rate of 0.5 Hz, using a diode-array UV detector (Mod. 168, Beckman Analytical). Identities of LTA_4-ME and LTA_4 free acid were assigned based on retention time and on-line UV absorbance spectra.

Metabolism of exogenous LTA_4 was allowed to proceed for 10, 20, 40, and 60 min at 37°C.

Metabolism of synthetic LTA_4 by lysed platelet preparations

Human or rabbit washed platelets were resuspended in lysis buffer (0.05 M phosphate buffer, pH 7.4, 0.1 M sodium chloride, 2 mM EDTA, 0.1 units/ml aprotinine, 1 g/ml pepstatin, 1 μg/ml leupeptin) at a concentration of $1–3 \times 10^9$ ml^{-1}, and sonicated 4 × 15 s on ice, using a sonifier (power setting 5, Mod. XL, Heat Systems, Farmingdale, NY) equipped with a microtip. Disrupted platelets were used either without further manipulation or centrifuged 20 min at 4°C and 10,000 *g*, and the supernatant was used for glutathione S-transferase activity determination. Proteins were quantitated

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spectrophotometrically using Coomassie Protein Assay Reagent (Pierce, Rockford, IL) with bovine serum albumin as standard. The equivalent of 10^8 platelets, from platelet lysates, was added to a reaction mixture (900 µl) containing 5 mM glutathione, 0.1 M phosphate buffer, pH 7.0, 1 mM EDTA (12). After 2 min of equilibration, LTA_4 free acid was added at a final concentration of 1 µM and the reaction was allowed to proceed for 1 min.

Incubations were terminated with 2 vol of ice-cold methanol containing the HPLC internal standard PGB_2 (25 ng) and samples were analyzed by RP-HPLC.

RP-HPLC analysis

Samples from cell incubations were diluted with water to a final methanol concentration lower than 20% and extraction was quickly carried out using a solid phase cartridge (Supelclean LC-18, Supelco, Bellafonte, PA); the retained material was eluted with 90% aqueous MeOH. After evaporation, the dried extract was reconstituted in HPLC mobile phase A, (see below) (600 µl) and injected into an HPLC gradient pump system (Mod. 126, Beckman Analytical) connected to a diode-array UV detector (Mod. 168, Beckman Analytical). UV absorbance was monitored at 280 and 235 nm, and full UV spectra (210–340 nm) were acquired at a rate of 0.5 Hz. An aliquot (600 µl) of samples from exogenous LTA_4 experiments was analyzed without prior extraction.

A multilinear gradient from solvent A (methanol–acetonitrile–water–acetic acid 10:10:80:0.02, (v/v/v), pH 5.5 with ammonium hydroxide) to solvent B (methanol–acetonitrile 50:50 (v/v) at a flow rate of 1 ml/min, was used to elute a 4 × 250 mm column (RP-18 endcapped Lichrospher, 5 µm, Merck). Solvent B was increased to 35% over 6 min, to 65% over 25 min, and to 100% over 3 min. This method allowed separation of LTB_4 from 5(S), 12(S)-dihydroxy-8,14-*cis*-6,10-*trans*-eicosatetraenoic acid (5,12-diHETE) as well as from Δ^6-*trans* LTB_4 isomers.

Positive identification of AA metabolites was obtained through UV spectral analysis of chromatographic peaks eluting at characteristic retention times. Retention times of standard compounds were: 20-OH-LTB_4, 12.5 min; LTC_4, 15.4 min; PGB_2, 17.5 min; Δ^6-*trans* LTB_4, isomers, 19.3 and 19.7 min; LTB_4, 20.5 min; 5,12-diHETE, 20.9 min; and 5,6-diHETE isomers, 24.4 and 25 min. Quantitation was carried out only on positively identified peaks, using their HPLC peak areas relative to that of PGB_2 at 280 nm, and calculated from the responses of standard compounds.

Assay of glutathione S-transferase activity

Glutathione S-transferase (E.C.2.5.1.18) activity in human and rabbit platelet cytosol (100–400 µg protein) was measured spectrophotometrically at 340 nm in a reaction system containing 1 mM glutathione, 1 mM CDNB, 5% ethanol, and 0.1 M sodium phosphate, pH 6.5, at 25°C (13). The increase in absorbance at 340 nm was monitored for 3 min using a Jasco spectrophotometer (V-530, Tokyo, Japan). Results were expressed as nmol of CDNB-glutathione conjugate formed per mg of protein per minute, using a molar extinction coefficient of 9600 $(mol/l)^{-1}$ cm^{-1} at 340 nm.

Data analysis

Amounts of LTA_4 metabolites were analyzed by analysis of variance (ANOVA) and Student's *t*-test.

Values were expressed as mean ± standard error of the mean (SEM) of n observations.

A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Challenge with the calcium ionophore A23187 (2 µM, 10 min) of peripheral rabbit mixed leukocyte preparations resulted in the production of LTB_4 (18.7 ± 2.1 pmol/10^6 cells, n = 5), LTC_4 (8.6 ± 0.8 pmol/10^6 cells, n = 5) and of non-enzymatic-LTA_4 metabolites (namely Δ^6-*trans*-LTB_4 isomers + 5,6 dihydroxy eicosatetraenoic acids); consistent with published data (8, 14), ω-oxidized LTB_4 metabolites, such as 20-hydroxy-LTB_4 (15) were not detected (**Fig. 1**). Minor amounts of 5(S), 12(S)-dihydroxy-8,14-*cis*-6,10-*trans*-eicosatetraenoic acid (5,12-diHETE), a metabolite arising from the sequential action of 5- and 12-lipoxygenase on the arachidonic acid (AA) (16), were also detected, indicating the presence of platelets as contaminant of the mixed leukocyte preparation (3.1 ± 1.6 platelets per leukocyte, n = 5). Addition of homologous platelets in a ratio of 40:1 with the leukocytes caused a significant increase of 5,12-diHETE and of LTB_4 while LTC_4 production did not change (Fig. 1 and **Fig. 2**). Similar results were obtained using purified polymorphonuclear leukocytes. Although LTB_4 production was higher than in mixed leukocyte preparations (42.3 ± 7.8 and 45.4 ± 4.2 pmol/10^6 cells, PMNL alone and PMNL + platelets, respectively; n = 3), only minimal amounts of LTC_4 were detected either in PMNL alone or in PMNL + platelets coincubations (1.9 ± 0.9 and 1.7 ± 0.8 pmol/10^6 cells, respectively; n = 3).

Challenge of peripheral human mixed leukocyte preparation with the calcium ionophore A23187 (2 µM, 10 min) resulted in the expected profile of LTA_4-derived metabolites (15). 20-Hydroxy-LTB_4 was the main product, with significant amounts of LTA_4, LTC_4, and non-enzymatic-LTA_4 metabolites. Presence of con-

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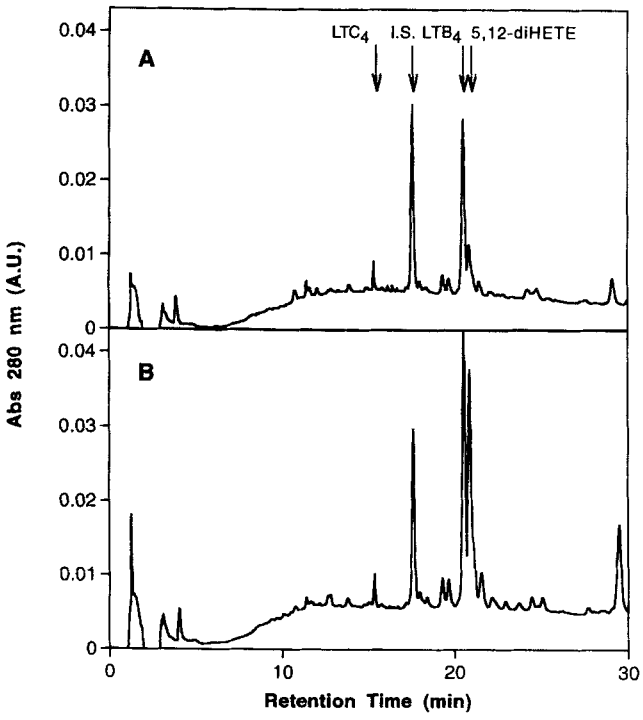

Fig. 1. LTA_4 metabolites synthesized by rabbit leukocytes and PMNL-platelet coincubations. UV absorbance profile at 280 nm from the RP-HPLC of rabbit leukocytes (5×10^6 cells ml^{-1}) (panel A) and leukocyte-platelet coincubations (ratio 1:40) (panel B) after challenge with the Ca^{2+}-ionophore A23187 (2 μM, 10 min, 37°C). Arrows indicate the retention time of synthetic standards. I.S.: internal standard (PGB_2).

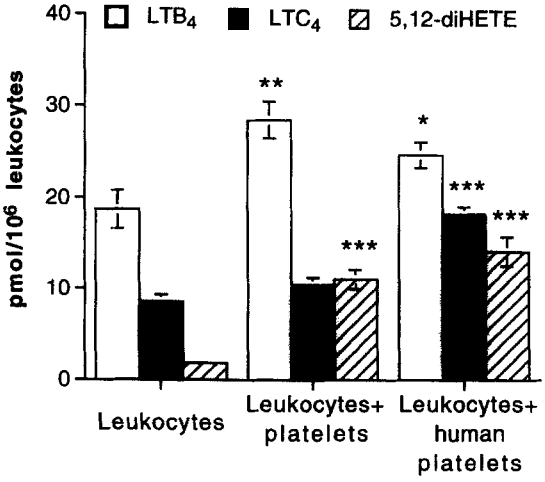

Fig. 2. Production of LTB_4, LTC_4, and 5,12-diHETE by mixed rabbit leukocyte preparations. Mixed rabbit leukocytes were challenged with the calcium ionophore A23187 (2 μM, 10 min, 37°C), with or without rabbit or human platelets in a ratio of 40:1 with the leukocytes. Values are expressed as pmol/10^6 cells. Means ± SEM of 5–7 different preparations.

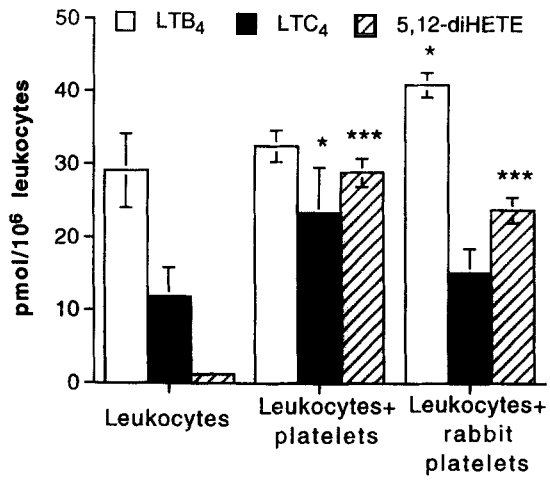

Fig. 3. Production of LTB_4, LTC_4, and 5,12-diHETE by mixed human leukocyte preparations. Mixed human leukocytes were challenged with the calcium ionophore A23187 (2 μM, 10 min, 37°C), with or without human or rabbit platelets in a ratio of 40:1 with the leukocytes. Values are expressed as pmol/10^6 cells. Means ± SEM of 5 different preparations.

taminating platelets (2.1 ± 1.3 platelets per leukocyte, n = 5) was unmasked by minor amounts of 5,12-diHETE. In agreement with previous data (4, 17), addition of homologous platelets resulted in a significant increase of LTC_4, together with the increase of 5,12-diHETE (**Fig. 3**).

In light of the results obtained with rabbit leukocyte-platelet coincubations, mixed preparations of human and rabbit blood cells were tested. Addition of human platelets, in a ratio of 40:1 with rabbit leukocytes, resulted in a 2-fold increase of LTC_4, showing that rabbit leukocytes were able to transfer intact LTA_4 to platelets. On the other hand, addition of rabbit platelets to human leukocytes resulted in a significant increase of LTB_4, as observed with rabbit leukocytes, but no changes were observed for LTC_4 (Figs. 2 and 3).

Human platelets were able to efficiently convert exogenous LTA_4 into LTC_4, in agreement with published data (4, 18). Rabbit platelets, in spite of prolonged incubations with different concentrations of LTA_4, were not able to synthesize amounts of cysteinyl leukotrienes as detectable by RP-HPLC (**Fig. 4**).

Human platelet lysates synthesized significant amounts of LTC_4 upon addition of 1 μM synthetic LTA_4 (114.3 ± 8.8 pmol/10^8 platelet equivalent). Disrupted rabbit platelets did not synthesize detectable amounts of LTC_4 (**Fig. 5**).

Glutathione S-transferase activity was 26.6 ± 9 (n = 9) nmol min^{-1} per mg of protein in human platelet lysates, and 9.3 ± 3 (n = 4) nmol min^{-1} per mg of protein in rabbit platelet lysates (19).

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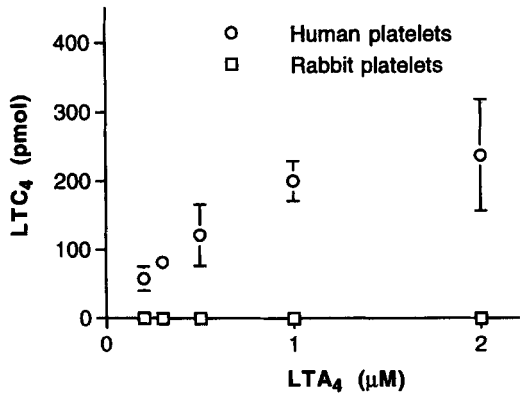

Fig. 4. Production of LTC_4 by human or rabbit platelets incubated with increasing concentrations of synthetic LTA_4. Human or rabbit platelets (10^8 cells) were incubated with increasing concentrations of synthetic LTA_4 free acid (0.2–2 μM, 10 min, 37°C) in presence of BSA (0.5%). Values are expressed as pmol. Means ± SEM of 3 different platelet preparations

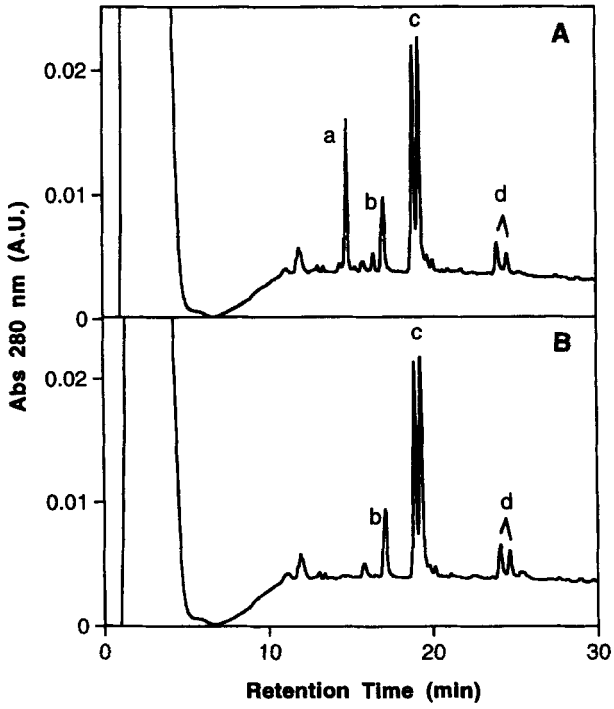

Fig. 5. LTA_4 metabolites synthesized by human and rabbit platelet lysates upon addition of synthetic LTA_4 free acid. UV absorbance profile at 280 nm from the RP-HPLC of human (panel A) and rabbit (panel B) platelet lysates (10^8 platelet equivalent) upon addition of synthetic LTA_4 free acid, as described in Experimental Procedures. Peaks are labeled as follows: a: LTC_4; b: PGB_2 (HPLC internal standard); c: Δ^6-*trans*-LTB_4 isomers; d: 5,6-diHETE isomers.

DISCUSSION

The importance of cell–cell interactions and cooperation for the production of biologically active leukotrienes requires the elucidation of the potential for transcellular metabolism of blood cells from the species most widely used in pharmacological research. The results reported in this study clearly show that rabbit leukocytes are able to export significant amounts of the unstable intermediate LTA_4, and therefore can participate as "donor cells" to transcellular biosynthetic processes involving "acceptor cells" other than platelets. Evidence of the cooperation of rabbit polymorphonuclear leukocyte with pulmonary endothelial cells for the production of cysteinyl leukotrienes has been presented by Grimminger et al. (7).

A recent report by Palmentier et al. (20), as well as data obtained in our laboratory (21, 22), suggest that intact LTA_4 might represent the main LTA_4-metabolite released by human or bovine PMNL. This important observation further enhances the potential for transcellular metabolism of LTA_4 as the main biosynthetic pathway for cysteinyl leukotriene biosynthesis in the presence of a targeted stimulus of polymorphonuclear leukocytes.

Although a very early report hypothesized that rabbit platelets were able to synthesize a slow reacting substance of anaphylaxis (SRS-A) from arachidonic acid (23), we did not not find any LTC_4 or 5-HETE after challenge of rabbit platelets with the calcium ionophore A23187, in agreement with a recent report providing the profile of arachidonic acid metabolites in rabbit platelets as analyzed by gas chromatography–mass spectrometry (24).

The study of LTC_4 formation in rabbit leukocyte-platelet coincubations showed that rabbit platelets, unlike human platelets, were not able to process PMNL-derived LTA_4 into the potent inflammatory mediator LTC_4. Intact rabbit platelets were not able to convert synthetic LTA_4 free acid into detectable amounts of LTC_4, suggesting that rabbit platelets do not have the specific LTC_4-synthase enzyme. In order to test whether this effect was due to the absence of a potential LTA_4 carrier, we also tested human and rabbit platelet lysates, and still no significant metabolism was observed in rabbit preparations.

Glutathione S-transferase activity showed substantial variability in platelet lysates and, although it appeared to be lower in rabbit platelets than that observed in human platelets, no statistically significant differences were observed.

Recent studies in complex organ systems showed that perfusion of PMNL in isolated rabbit lung or heart resulted in production of significantly increased amounts of cysteinyl leukotrienes only when PMNL were activated during the perfusion process (7–10). This increase was accompanied by significant functional and morphological modifications, suggesting that transcel-

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lular biosynthesis of cysteinyl leukotrienes might indeed be of physiopathological relevance when tight cell–cell interactions occur, such as during adhesion and diapedesis of PMNL through the microvascular endothelium of a functioning organ system. In agreement with this hypothesis, a specific leukotriene synthesis inhibitor (LSI), BAY X1005, was able to significantly improve survival after permanent ligature of the left descending coronary artery in the rabbit (10). The data in the present study clearly show that rabbit platelets, unable to synthesize LTC_4 from LTA_4, cannot play an important role in the biosynthesis of cys-LT that underlies the cardiac damage and the related mortality after coronary ligature. Vascular endothelial cells seem to be the preferential "acceptor cells" in the mechanisms of transcellular biosynthesis of cys-LT in the rabbit, in agreement with the in vitro observations using both human and rabbit PMNL to perfuse isolated rabbit heart or lung.

Platelet–leukocyte interactions involving the production of lipoxygenase products are of potential significance in humans for the inflammatory process, hemostasis, and thrombosis, particularly in light of the fact that they are aspirin-insensitive interactions (25–27), and therefore special care must be taken using the rabbit as an experimental model.

Large amounts of the double oxygenation product 5,12-diHETE were observed in leukoycte–platelet coincubations, in agreement with published data (16) and according to the sequential action of leukocyte 5-LO and platelet 12-LO on arachidonic acid, with transfer of either 5-HETE or 12-HETE from one cell to the other.

In purified rabbit PMNL preparations, LTB_4 production was about 2-fold higher than in mixed leukocyte preparations, where PMNL might represent up to 70% of the total leukocytes. Therefore, the observed increase was not merely due to the increased number of PMNL, and suggested that the autocrine effect of LTB_4 and platelet-activating factor (PAF) on neutrophils 5-LO observed in purified PMNL preparations (28) may be significantly blunted in mixed leukocyte preparations.

A significant increase in LTB_4 production was observed upon addition of platelets to mixed leukocyte preparations, but not to purified rabbit PMNL preparations. These results are in agreement with those of Maclouf et al. (29) and Antoine et al. (30), obtained using very similar experimental conditions. 12-Hydroperoxyeicosatetraenoic acid (12-HpETE), derived from platelet arachidonic acid oxidative metabolism, has been shown to activate the human blood leukocyte 5-lipoxygenase (29), and therefore is a likely candidate to account for the enhanced LTB_4 production in platelet–mixed leukocyte coincubations. The lack of further enhancement of LTB_4 production in purified PMNL-platelet coincubations seems to suggest that the autocrine activation of 5-LO by LTB_4 is not additive with that of 12-HpETE.

In conclusion we show that rabbit PMNL are able to export intact LTA_4, while rabbit platelets, unlike human or bovine platelets, are not able to metabolize LTA_4 into LTC_4 to a significant extent. The results of the present study provide important information for the proper design and/or interpretation of research involving the rabbit as an experimental model.

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