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BLOOD LEVELS OF LEUKOTRIENES (LTC_4, D_4, E_4, B_4) AND SYNTHESIS OF LEUKOTRIENE B_4 BY PERIPHERAL LEUKOCYTES IN CHILDREN WITH ACUTE A AND B HEPATITIS*

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SUMMARY: Kasırga E, Çoker I, Aydoğdu S, Yağcı RV, Taneli B, Gousseinov A. (Division of Gastroenterology, Department of Pediatrics, Ege University Faculty of Medicine, İzmir Turkey). Blood levels of leukotrienes (LTC_4, D_4, E_4, B_4) and synthesis of leukotriene B4 by peripheral leukocytes in children with acute A and B hepatitis. Turk J Pediatr 1999; 41: 457-465.

Leukotrienes (LTs) are cell-membrane derived lipid inflammatory mediators, synthesized and eliminated by the liver. LTs have effects on liver cells in some pathological conditions. In this study, we measured plasma endogenous and liberated leukotriene (LT) concentration in peripheral blood leukocytes stimulated in vitro by the calcium ionophore (CaA23187) and platelet-activating factor (PAF). Production of LTs was measured in type A (n=37) and type B (n=10) acute hepatitis patients and control subjects (n=10). LTs levels were measured by high performance liquid chromatography (HPLC) and radioimmunoassay (RIA). The concentration of LTB_4 measured in plasma and stimulated peripheral blood leukocyte supernatants of children with hepatitis A infection was found to be statistically elevated and in positive correlation with serum alanine aminotransferase (ALT) levels. In plasma samples of hepatitis B patients, LTC_4 and LTE_4 were measured in significantly elevated concentrations. These results suggest that LTB_4 may be a critical mediator of hepatitis A virus-induced hepatocellular injury.
Key words: leukotrienes, lipid mediators, acute viral hepatitis.

Leukotrienes (LTs) are lipid inflammatory mediators that have important roles in inflammation and anaphylactic reactions[1]. These mediators are derived from arachidonic acid, released from cell membranes and during the 5-lipoxygenase pathway[2]. LTs are synthesized and released from cells such as neutrophils, monocytes, macrophages, mast cells, eosinophils, platelets, lymphocytes, Kupffer cells, renal glomerular cells, gastric epithelial cells and vascular endothelial cells[3,4]. Peptide LTs lead to edema and inflammatory cell infiltration caused by vasoconstriction and increased venopermeability at the inflammation site[1,2,5]. LTB_4, a nonpeptide leukotriene (LT), is known to be a potent chemotactic agent[1,2]. LTB_4 is responsible for activation, chemotaxis and degranulation of

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inflammatory cells[6]. At the end of these reactions, free O_2 radicals and proteases, cytokines, cationic proteins, serotonin and thromboglobulin are released from neutrophils, monocytes, lymphocytes, eosinophils and platelets[2,6]. Many laboratory studies have been performed during recent years in rats. CCl_4 or galactosamine with lipopolysaccharides and frog virus-3 were used for the induction of toxic and viral hepatitis, respectively, in rats[7-9]. It has been shown that liver destruction can be prevented using LT synthesis inhibitors or LT receptor antagonists[10,11]. LTs undergo omega and beta oxidation by N-acetylation in the liver parenchymal cells and then are excreted in bile[12]. LTs are synthesized in the liver during different pathological conditions. It is known that the liver is the target organ for many of these biologically active mediators[3].

The aim of this investigation was to study the role of LTs in the pathophysiologic mechanisms in hepatitis A and hepatitis B infections.

Material and Methods

Forty-seven patients with acute viral hepatitis (37 hepatitis A; 10 hepatitis B) seen in the outpatient Department of Pediatrics at Ege University School of Medicine were studied. The male/female ratios were 19/18 and 5/5 for the hepatitis A and B groups, respectively. The patients' mean ages (mean±SD)' were 8.67±2.40 and 11.2±3.52 years for hepatitis A and hepatitis B virus infections, respectively. All patients had acute hepatitis symptoms. There were no clinical or laboratory findings that suggested chronic liver disease in this group.

Biochemical and Serological Analysis: Liver function tests, anti-HAV IgM, anti-HAV IgG, HBsAg, anti-HBc IgM, anti-HBc total, HBeAg, and anti-HBe were assayed in all patients. Anti-HAV IgM (+), HBsAg (+) and anti-HBc IgM (+) patients were included in the study. Serological tests were performed by an ELISA method (Organon, Boxtel, Holland). Serum alanine aminotransferase (ALT) levels were measured on Hitachi 911 autoanalyzers by the Chod PAP enzymatic method.

Leukotriene Extraction from Plasma: One ml fresh plasma was separated from heparinized blood that was mixed with cold phosphate buffered saline without calcium at a 1:10 ratio and then centrifuged at 5,000 rpm for 10 min at + 4 °C. LTs were extracted from plasma using Maxi Clean C18 600 mg columns (Alltech, Co., USA) which had been conditioned and preactivated with 20 ml of methanol and then 20 ml of distilled water. The eluate was collected after applying 2 ml of acetonitrile solution and then passed through a 0.22 µ cellulose membrane filter (Alltech, Co., USA) in evaporator tubes. The acetonitrile solution was evaporated to dryness by a vacuum speed concentrator. The residue was dissolved in 20 µl of high performance liquid chromatography (HPLC) solution and stored at -70 °C until testing.

Leukocyte Isolation from Peripheral Blood: 10 ml venous blood was collected in a heparinized tube at a 0.01:10 ratio of heparin: blood and then a 10 percent gelatine solution was added to the blood at a 1:0.15 ratio of gelatin solution: blood. This mixture was incubated at 37 °C for 45 min. The cells were obtained from the lukocyte-rich plasma which settled as the upper phase by centrifugation at + 4 °C at 1,500 rpm for 7 min. The cells were then resuspended in HANKS balanced salt solution without Ca^{++} and Mg^{++} and washed twice. Finally, the cells were resuspended in 2 ml HANKS solution and a 20 µl cell suspension aliquot was taken out and diluted 3% acetic acid solution at a 1:20 ratio and counted in a Neubauer Chamber. The cell suspension was adjusted to a concentration of $1x10^7$ cells per milliliter in HANKS solution with Ca^{++} and Mg^{++}. The cell suspension was divided into two tubes for either calcium ionophore (CaA23187) or platelet-activating factor (PAF) stimulation. Leukocytes were incubated at 37 °C in an ultrasonic bath with 20 µl of 5 µm calcium ionophore for 15 min and 10 µl of 1 µm synthetic PAF C16 for 10 min. Incubations were stopped by adding 1 ml of cold deionized water and the leukocyte suspension was centrifuged immediately at + 4 °C at 6,000 rpm for 3 min. LT extraction was performed from the supernatant; the leukocyte sediment was discarded.

Leukotriene B_4 Extraction from Stimulated Leukocyte Supernatant: 0.5 ml of 2-propanol and 30 µl of 5 M formic acid were added to 1 ml of the supernatant from the leukocyte stimulation experiments and incubated for 10 min. Ether (1.5 ml) was then added dropwise and the solution mixed well and left until the appearance of two phases. The upper ether phase was removed and 22 µl of NH_3 was added to it. This mixture was poured into the microcentrifuge system (Alltech, Co., USA). A 0.22 µ diameter membrane filter was placed on the filtration part of this system. After centrifugation for 5 min, the filtrate which passed through the lower tube was dried by a vacuum speed concentrator and then stored in 20 µl HPLC solvent at -70 °C until testing.

Purification of Leukotrienes by the HPLC Method: Analysis of LTs was performed using the Waters 625 LC System. This system consisted of a four channel multidelivery pump, Waters 486 tunable absorbance UV detector, powerline system controller, Rheodyne 7012 injector with 20 µl sample loop and Baseline 810 HPLC software program. LT separation was performed on a Separon SG X C18 super analytical column (250x2.0 mm I.D) and precolumn (100x2.0 mm I.D) with a (methanol: water: acetic acid) gradient measuring at 280 nm wavelength and a 1.5 ml/min flow rate. LTs were collected from HPLC UV detector output according to their retention times and were dried again in the vacuum speed evaporator. Retention times were 7', 11', 13' and 15' for LTE_4, LTB_4, LTD_4 and LTC_4, respectively. The amounts of LTB_4, LTC_4, LTD_4 and LTE_4 were quantitatively measured by using LTB_4 (^3H), LTC_4 (^3H), LTD_4 (^3H) and LTE_4 (^3H) assay systems

(Amersham Life Science, UK). The procedure was performed according to kit instructions. LTB_4, LTC_4, LTD_4 and LTE_4 concentrations were measured by Beta-liquide scintillation counter (TRI-CARB-1600 TR, LSA-Packard, Canberra Company).

Results of LTs were expressed as ng/ml and ng/10^7 cells in plasma samples and peripheral leukocyte secretions, respectively.

Statistics: Data on LT concentrations and ALT values are expressed as mean, standard error and ranges. Differences found between the patients and controls were analyzed by Student's two-tailed *t* test. Correlation coefficients were calculated with the Spearman rank test. A p value of less than 0.05 was considered significant.

Results

Endogenous LTs concentrations were determined in the plasma samples from 23 of our 37 children with hepatitis A infection. Released LTB_4 concentration was evaluated in the supernatants of PAF-stimulated peripheral blood leukocytes from 36 of our 37 hepatitis A patients. Produced and released LTB_4 concentration was examined in the supernatants of CaA23187 stimulated leukocytes obtained from 29 of 37 hepatitis A patients. Endogenous LTs and released LTB_4 were determined in the plasma and stimulated leukocyte supernatants from all of the 10 patients with acute hepatitis B infection. In contrast to the hepatitis patients, normal subjects had almost undetectable blood levels of LTs. After stimulation with CaA23187, release of LTB_4 was observed in healthy subjects. On the other hand, in PAF-stimulated healthy subjects' leukocyte supernatants, LTB_4 was undetectable. In plasma, LTC_4 was untedetectable in 96.5 percent (28/29) of samples of children with hepatitis A and in 10 percent (1/10) of samples of patients with hepatitis B infection. Plasma samples of LTD_4 levels were undetectable in 100 percent (29/29) of patients with hepatitis A and in 70 percent (7/10) of patients with hepatitis B.

There were significant differences in the endogenous plasma LTs concentrations in the hepatitis A and B groups. LTB_4 was higher in plasma samples of patients with hepatitis A compared with those with hepatitis B ($t=2.15$, $p<0.05$). In plasma samples, LTC_4 and LTE_4 concentrations in children with type B hepatitis were found to be significantly higher when compared to patients with type A hepatitis ($t=6.55$, $p<0.001$ and $t=3.64$, $p<0.001$, respectively). Levels of LTD_4 were low and not different between type A and B hepatitis groups (Table I).

In the type A hepatitis group, it was shown that stimulation by CaA23187 and PAF yielded higher LTB4 release from peripheral leukocytes than in the hepatitis B group ($t=4.48$, $p<0.001$ and $t=2.37$, $p<0.05$, respectively) (Table II).

Table I: Concentrations of LTs in Plasma Samples (Mean±SEM)

Leukotrienes	Hepatitis A Group (n=29)	Hepatitis B Group (n=10)	p
LTB_4	1.8±0.1 (0.48-3.46)	1.1±0.6 (0.6-2.1)	<0.05
LTC_4	0.02±0.02 (0-0.64)	0.6±0.1 (0-1.93)	<0.001
LTD_4	ND	0.1±0.07 (0-0.61)	NS
LTE_4	1.5±0.1 (0.46-2.9)	2.4±0.2 (0.9-3.6)	<0.001

Leukotrienes values are expressed as ng/ml, (range).
ND: non-detectable, NS: not significant.

Table II: Concentration of LTB4 in the Supernatant of PAF and CaA23187-Stimulated Peripheral Blood Leukocytes (Mean±SEM)

LTB_4	Hepatitis A Group	Hepatitis B Group	p
	(n=36)	(n=10)	
PAF	9.7±0.8 (2.1-19.4) (n=30)	5.7±0.5 (3.9-9.2) (n=10)	<0.05
CaA23187	32.5±1.5 (18.4-49.6)	20±1.2 (14.2-26.4)	<0.001

Leukotrienes values are expressed in ng/107 cells, (range).
PAF: platelet-activating factor.

LTB_4 production was significantly higher in CaA23187 stimulated peripheral blood leukocytes from patients associated with hepatitis A and B virus infections compared with healthy control subjects ($t=11.07$, $p<0.001$ and $t=14.07$, $p<0.001$, respectively). Serum ALT levels in patients with hepatitis A (mean±SEM: 1369.7±115.1, range: 593-3312) were found to be significantly higher than in children with hepatitis B (mean±SEM: 732.6±172.9, range: 83-1811) ($t=2.65$, $p<0.05$). In the type A hepatitis group, there was a statistically significant positive correlation between serum ALT levels and the plasma concentrations of LTB_4 and LTB_4 release from peripheral leukocytes after in vitro PAF stimulation ($r=0.41$, $p<0.05$ and $r=0.44$, $p<0.01$, respectively). A significant correlation was not found between serum ALT levels and secreted LTB_4 concentrations from peripheral leukocytes after CaA23187 stimulation. On the other hand, there was no statistically significant correlation between serum ALT levels and LTs in the hepatitis B group.

Discussion

A considerable fraction of the LTs formed intrahepatically or systemically is excreted in the bile, but a portion gets into the circulation[11,13]. LTs which are in the circulation are either released from the liver or formed by further metabolization in the plasma. In the plasma, LTD_4 is formed from LTC_4, and LTE_4 is formed from LTD_4. Therefore, the fact that the level of LTE_4 determined in the plasma in both types of hepatitis was higher compared to the concentrations of LTC_4 and LTD_4 can be explained by accumulation of the end-product (Table I). By activating the peripheral blood leukocytes, the LTs synthesized in the liver and released into the circulation can initiate secretion of LTs also from cells. Accordingly, it may be wrong to say that the source of the endogenous LTs determined in the plasma is solely the liver. Thas is, these mediators are released both by the liver and the peripheral blood leukocytes. The difference between the activities of the enzymes that take part in the synthesis and metabolism of LTs and the effects of the hepatitis viruses on Kupffer cells and hepatocytes may cause the different LT concentrations in the plasma of type A and B hepatitis patients. Differences between these enzyme activities have been demonstrated in mice in the studies of Kawada and collaborators[14]. It is thought that in hepatitis A and B, the peripheral blood leukocytes participate in the inflammatory reaction by infiltrating into the liver and that they then pass into the peripheral circulation, having been sensitized. This contribution happens by a secretion of biologically active substances, especially by cells in the activated inflammation zone. During hepatitis it is shown that local mediators are secreted by activated Kupffer cells[15,16]. This secretion increases even more during viral stimulation. In 1987 Hagmann et al.[8] stimulated Kupffer cells which they had isolated with in vitro FV-3 (Frog virus 3) and they showed that both peptide and non-peptide LTs were secreted in large amounts. Additionally, they determined that the LT receptor antagonists which were used in vivo and in vitro inhibited the hepatocellular destruction that occurs as a result of the virus effect. The different amounts and spectrum of LT secretion, depending on the type of inflammation and as a result of the in vitro stimulation of leukocytes obtained from patients having different inflammatory reactions, has been shown[17-19]. PAF is a powerful otocoid mediator that plays a very important role in all stages of immune and infectious inflammation, and it is made from the cell membrane[20,21]. It is known that PAF is secreted in the liver by Kupffer cells and that at the same time it is effective on the liver[22,23]. LTB_4 and peptide LTs are formed with PAF and they potentiate the effects of each other[3]. Aiming to show that LTs are secreted as a result of the effect of PAF and to simulate the effects of these secreted LTs on the liver under in vitro circumstances, the peripheral leukocytes obtained from our patients of both hepatitis groups were stimulated by PAF. After doing so, very interesting differences are observed (Table II).

LTB_4 and PAF, very strong chemotactic factors released in the intrahepatic region, have different cells migrated into the liver. These infiltrated cells can lead to the release of a local mediator by degranulation, or pass into the peripheral circulation in an active form and then activate other intact cells by intracellular PAF or LT.

Inflammatory reactions are based on an increase of intracellular calcium and include LT synthesis by changing the composition of membrane phospholipids. The CaA23187 also caused LTB_4 secretion from peripheral leukocytes obtained from healthy children as a control group. Leukocytes from hepatitis patients in vitro secrete active LTB_4 when stimulated by CaA23187 (Table II). It can be concluded that membrane calcium channels are very active in peripheral leukocytes in patients with acute viral hepatitis.

Serum liver enzyme levels increase rapidly just after the prodromal period in viral hepatitis. Increased liver enzyme levels could be used as an indicator of cell damage[24]. Serum ALT levels, plasma LTB_4 concentrations and LTB_4, which were secreted from peripheral blood leukocytes after PAF treatment, were correlated to each other in hepatitis A and these correlations were statistically significant. However, in patients with hepatitis B there was no statistically significant correlation between serum ALT levels and LTs. It can be speculated that hepatitis B can cause slow pathogenic reactions while hepatitis A may cause rapid reactions. This can be a leading factor for progressive chronic viral hepatitis B, among the many factors responsible. Interferon (IFN) synthesis is one of the host originated and intracellular factors affected by LTB_4. Other factors like decreased MHC-II expression and IFN activity in chronic viral hepatitis B can cause defects in synthesis or secretion of LTB_4. It can be claimed that decreased amounts of MHC-II type receptors, which are necessary in the production of anti-HBs antibodies, can cause problems in amplification of immune reactions by decreased interactions between T and B cells. Anti-HBs antibody synthesis, the antibody responsible for extracellular virus clearance, can decrease at the end of these reactions[25,26].

A major difference between LTs concentrations in hepatitis A and B patients may be an important prognostic indicator. A high LTB_4 response in hepatitis A may prevent chronic hepatitis A infections. Serum ALT levels and chemotactic LTB_4 originating from PAF-stimulated leukocytes and plasma are correlated in hepatitis A and this finding indicates the above-mentioned assumption can be considered. This difference between hepatitis A and B may be related to the cytopathic effects of the hepatitis A virus.

We thus conclude that a high response rate LTB_4 secretion by peripheral leukocytes to stimulants like CaA23187 and PAF and correlation between either endogenous or PAF-stimulated LTB_4 and ALT levels suggest that LTB_4 has a very important place in A type acute viral hepatitis pathophysiology and clinical course.

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