Biochemical Pharmacology, Vol. 37, No. 16, pp. 3193–3197, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

METABOLISM OF T-2 TOXIN BY BLOOD CELL CARBOXYLESTERASES

HELGE JOHNSEN, ERLING ODDEN, BJØRN A. JOHNSEN and FRODE FONNUM
Norwegian Defence Research Establishment, Division for Environmental Toxicology, N-2007 Kjeller, Norway

(*Received* 3 *December* 1987; *accepted* 24 *March* 1988)

Abstract—Human and rat blood hydrolysed T-2 toxin along two different pathways giving HT-2 toxin and neosolaniol as primary metabolites, respectively. Neosolaniol represents a metabolic pathway different from that obtained by liver. Rat erythrocytes formed neosolaniol as a primary metabolite whereas white blood cells hydrolysed T-2 toxin to HT-2 toxin. Human erythrocytes formed both HT-2 toxin and neosolaniol whereas all human white cells produced only HT-2 as the primary metabolite. The enzymes responsible for hydrolysis of T-2 toxin to HT-2 toxin in white blood cells and T-2 toxin to neosolaniol in red blood cells were all identified as carboxylesterases by use of specific inhibitors. The ratio between trichothecene hydrolysis and 4-nitrophenyl butyrate hydrolysis varied among the different cell fractions indicating that specific isoenzymes are involved.

T-2 toxin is one of the most important trichothecene mycotoxins occurring naturally in agricultural products and is associated with several characteristic mycotoxicoses in both humans and animals.Red mold toxicoses in Japan, moldy corn toxicoses in the U.S.A. and alimentary toxic aleukia (ATA) in Europe are diseases caused by trichothecene mycotoxins [1, 2]. Furthermore, trichothecene mycotoxins have attracted international attention because of their possible use as the chemical warfare agent "Yellow Rain" [3–5].

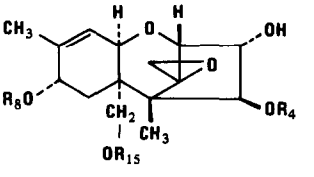
The metabolic activity in different tissues have been studied in rats and rabbits by Ohta *et al.* [6]. In addition to the liver, both kidney and brain were found to hydrolyse T-2 toxin to HT-2 toxin to some extent, whereas no activity was found in plasma or blood cells.

T-2 toxin (Fig. 1) is metabolized in liver mainly by hydrolysis at C-4 position by carboxylesterase to yield HT-2 as its main metabolite [6, 7]. When rat liver carboxylesterases were separated into five different isoenzymes, only isoenzyme pI 5.4 did hydrolyse T-2 to HT-2 toxin [7]. A different pathway possible for hydrolysis of T-2 toxin consists of removal of the isovaleryl group at C-8 position to yield neosolaniol, and this has been reported as a minor pathway in rat and mice intestine [8, 9]. The authors proposed that the hydrolysis to neosolaniol is caused by esterases of dermal microflora. Furthermore, neosolaniol has also been identified as a trace metabolite in mice [9] and swine [10]. T-2 toxin has also been found to be hydroxylated at 3-position of C-8-isovaleryl group by liver cytochrome P-450 [11].

In a previous study at our laboratory we found evidence that T-2 toxin was metabolized by carboxylesterase [7]. Cytochemical methods for identification of monocytes and granulocytes consist of staining for non-specific esterases [12]. Furthermore, multiple forms of esterases with different biochemical characteristics have been identified in the erythrocytes [13, 14]. The presence of esterases in the blood cells, indicates that T-2 toxin to some extent may be hydrolysed in the blood. In the present study we have, therefore, studied the hydrolysis of T-2 toxin by rat and human blood cells.

MATERIALS AND METHODS

Chemicals. T-2 toxin, HT-2 toxin, T-2 tetraol, Neosolaniol, Diacetoxyscirpenol (DAS), bis-4-nitrophenyl phosphate (BPNP), and physostigmine were all purchased from Sigma Chemical Company (St. Louis, MO). Paraoxon came from Koch-Light Laboratories, U.K. 4-Hydroxymercury benzoate-(4OH-MB) were purchased from Aldrich Chemical Company, Inc. (U.S.A.), whereas EDTA came from E. Merck (Darmstadt). Sylon



TRICHOTHECENE	R_4	R_8	R_{15}
T-2	CH_3CO	$(CH_3)_2CH\ CH_2CO$	CH_3CO
HT-2	H	$(CH_3)_2CH\ CH_2CO$	CH_3CO
4-DEACETYL-NEOSOLANIOL	H	H	CH_3CO
T-2 TETRAOL	H	H	H

Fig. 1. Chemical structure of the trichothecene T-2 toxin and its hydrolytic metabolites.

BTZ from Supelco, Inc. (PA, U.S.A.) was used as trimethylsilyl derivatizing reagent for gas chromatography mass spectrometry. Nycodenz (1.090 g/ml, 309 mOsm) from Nycomed A/S, Norway, was used for cell separation.

Animals and blood sampling. Male Wistar rats (200–300 g) of the outbred strain Mol: WIST were used in this study. They were maintained at constant room temperature (24°) with 12 hr light cycle and standard laboratory diet and water *ad libitum*. Blood was collected by puncture of the vena cava in animals anesthetized with diethyl ether, and anticoagulated with 3.8% sodium citrate (5:1 v/v).

Preparation of blood cells. Differential preparation of the blood cells was performed by methods previously developed in our laboratory [15–17]. The purity of the cell fractions was examined microscopically and found to be more than 90% for erythrocytes, white blood cells, lymphocytes and granulocytes. Leucocytes and erythrocytes were virtually absent in the thrombocyte fraction.

Whole blood. Rat and human blood was anticoagulated with 3.8% Na–citrate (5:1 v/v). Plasma was obtained by centrifugation of whole blood at 800 *g* for 15 min.

Red blood cells (RBC). The plasma free cells were washed 4 times with 0.9% NaCl and careful removal of the upper layer with the white blood cells with a pipette. The RBC was resuspended in 50 mM Tris(0.7% NaCl) pH 7.4.

White blood cells (WBC). Anticoagulated whole blood was mixed with Dextran (6%, T500, MW 530 000 in 0.9% NaCl) at a ratio 5:1. The RBC was allowed to sediment at room temperature for 30 min before the plasma residue containing the WBC was collected with a pipette.

Lymphocytes and granulocytes. The dextran treated plasma was layered over an equal volume Nycodenz (1.090 g/l, 309 mOsm) and centrifuged at 600 *g* for 15 min. The mononuclear cells were concentrated in a white layer at the interface region and when isolated from rat blood this layer consisted mainly of lymphocytes together with platelets. The sediment after centrifugation contained the granulocytes, and was resuspended in 1.2 ml 50 mM Tris/0.7% NaCl pH 7.4.

Thrombocytes. Anticoagulated whole blood was centrifuged at 600 *g* for 10 min. The plasma was recentrifuged at 800 *g* to sediment the thrombocyte fraction which was washed once and resuspended in 50 mM Tris/0.7% NaCl pH 7.4 before counting.

Monocytes. Monocytes from rats anesthetized until death with ether were harvested from peritoneal cavity by washing the peritoneum with 10 ml 0.9% NaCl. The ascites fluid was centrifuged at 800 *g* for 10 min and the cells resuspended in 1.2 ml 50 mM Tris/0.7% NaCl pH 7.4. The monocytes/macrophages constitute about 60–80% of the cell population in the ascites fluid.

Incubation of T-2 toxin. To aliquots of 1 ml of blood, plasma or cell preparations were added 0.5 μmole T-2 toxin and incubated for 1 hr at 37° and pH 7.5. Preincubation with enzyme inhibitors was performed at the same conditions for 30 min. The reaction was stopped by adding 2 ml ice-cold 0.6 M perchloric acid and 50 μg DAS as internal standard. Preparation of the samples is described in a previous study [7]. Trimethylsilyl derivatisation for gas chromatography was performed by Sylon BTZ.

Gas chromatographic mass spectrometric analysis. Gas chromatography was performed by a Packard Becker 438 gas chromatograph connected to a LKB 2091 mass spectrometer for identification and quantification of trichothecene metabolites. A glass column (1.2 m × 2 mm I.D.) packed with 3% OV-17 on Supelcoport 80–100 mesh was used under the following conditions: oven temperature programmed from 200° to 275° at 15°/min, injector and interface temperature 300° and flow rate of helium as carrier gas was 30 ml/min. The operating conditions for mass spectrometry were: ion source temperature 270°, ionization energy 70 or 14 eV and 22 eV for multiple ion detection (MID) and acceleration voltage 3.5 kV. Quantification of metabolites was performed by hardware multiple ion detection (MID) with DAS as internal standard. 4-deacetyl neosolaniol was not available as standard and quantification of this metabolite was therefore based on the response factor of neosolaniol.

RESULTS

T-2 toxin was completely hydrolysed to polar metabolites in 24 hr when incubated with rat blood. Table 1 shows the time course of the hydrolysis of T-2 toxin. It is of special interest to note that neosolaniol is one of the major metabolites produced in blood. In fact, the neosolaniol production was 62 ± 3% (SD, N = 3) of that of HT-2 toxin during the first 3 hr of incubation, and at 24 hr the neosolaniol content was 20% higher than HT-2 toxin. Both HT-2 toxin and neosolaniol reached maximum levels around 3 hr. These metabolites were both subjected to further hydrolysis to the more polar compounds 4-deacetyl neosolaniol and T-2 tetraol. T-2 toxin was metabolized to the same extent by human and rat blood. Formation of the polar metabolites 4-deacetyl neosolaniol and T-2 tetraol, however, was more pronounced in the human blood (Table 2).

Both erythrocytes and white blood cells from rat and human blood hydrolysed T-2 toxin. In rat blood, however, the hydrolysis of T-2 toxin was completely separated into the two different pathways by sep-

Table 1. Time course of the hydrolysis of T-2 toxin in rat blood

Time (hr)	Metabolite[a] (molar % of added substrate)				
	T-2[b]	HT-2	NEOS	4-DANS	T4
0.5	87	7	5	2	0
1	74	13	8	6	0.2
3	36	22	14	27	2
24	0	7	8	55	31

[a] Abbreviations: NEOS, neosolaniol; 4-DANS, 4-deacetyl neosolaniol; T4, T-2 tetraol. Values are means of triplicate experiments.

[b] Unmetabolized substrate after incubation of 540 μmole·ml^{-1} T-2 toxin.

Table 2. Hydrolysis of T-2 toxin by rat and human blood cells

Fraction		Cells·ml^{-1}	Metabolite[a] (nmole·ml^{-1}·hr^{-1})				
			T-2[b]	HT-2	NEOS	4-DANS	T4
Whole blood	Rat		358 ± 4	103 ± 2	52 ± 3	53 ± 6	tr[c]
	Human		303 ± 8	85 ± 7	50 ± 3	71 ± 15	16 ± 5
RBC	Rat	$6.4 \pm 0.2 \cdot 10^9$	483 ± 36	0	81 ± 5	11 ± 1	0
	Human	$4.9 \pm 0.4 \cdot 10^9$	315 ± 36	66 ± 9	45 ± 5	44 ± 6	10 ± 1
WBC	Rat	$21.4 \pm 0.1 \cdot 10^7$	365 ± 28	123 ± 28	tr	12 ± 2	0
	Human	$26.9 \pm 1.3 \cdot 10^7$	421 ± 56	28 ± 7	tr	tr	0

[a] Abbreviations: NEOS, neosolaniol; 4-DANS, 4-deacetyl neosolaniol; T4, T-2 tetraol. Values are mean ± SD, N = 3.
[b] Unmetabolized substrate after incubation of 540 nmole·ml^{-1} for 60 min.
[c] tr, trace amount.

aration into the red and white blood cells. Rat erythrocytes formed neosolaniol as the primary metabolite whereas white blood cells hydrolysed T-2 toxin to HT-2 toxin (Table 2). This specific hydrolysis of T-2 toxin in rat erythrocytes to give only neosolaniol as primary metabolite was not found for human erythrocytes, which produced HT-2 toxin and neosolaniol in equal amounts.

Previous studies in liver showed that T-2 toxin was metabolized by carboxylesterases [7]. There were, however, considerable differences towards T-2 toxin by the different carboxylesterase isoenzymes. It was therefore deemed necessary to characterize the metabolic activities possessed by red and white blood cells by use of specific esterase inhibitors which is presented in Table 3. The activities were completely blocked by the serine esterase inhibitors paraoxon (10^{-4} M) and soman (10^{-5} M), but only partly inhibited by the carboxylesterase inhibitor bis-4-nitrophenylphosphate (BPNP). In fact, the T-2 to HT-2 hydrolytic activity in white blood cells were inhibited by 34% by 10^{-4} M BPNP whereas no inhibition of T-2 to neosolaniol hydrolysis in red blood cells was seen. A concentration of BPNP at 10^{-3} M was necessary to inhibit the T-2 to neosolaniol hydrolysis to some degree. This low affinity to BPNP was, however, also found for the carboxylesterase isoenzyme hydrolysing T-2 toxin in liver [7]. Furthermore, no effect was seen with EDTA (10^{-3} M), an arylesterase inhibitor [18, 19] or 4-hydroxy mercurybenzoate (10^{-3} M) which inhibits esterases with SH-group in active site, such as phosphoryl phosphatases [20]. Finally the hydrolysis was not affected by physostigmin at 10^{-5} M, a concentration known to specific inhibit cholinesterase whereas carboxylesterase is not inhibited. The enzyme responsible for the hydrolysis of T-2 toxin in blood cells therefore had similar properties as the carboxylesterase in liver hydrolysing T-2 toxin.

When T-2 toxin was incubated with isolated preparations of monocytes, lymphocytes, granulocytes and thrombocytes, which makes the white cell population in blood, it was found that all these different cell types participated in the hydrolysis of T-2 toxin to HT-2 toxin (Table 4). No neosolaniol was produced by these cells. The different cell populations did also hydrolyse the well defined substrate for carboxylesterase; 4-nitrophenylbutyrate, indicating

Table 3. Effect of esterase inhibitors on hydrolysis of T-2 toxin to HT-2 toxin by white blood cells and T-2 toxin to neosolaniol by erythrocytes

Inhibitor	Metabolite produced (% of control)	
	Neosolaniol (erythrocytes)	HT-2 toxin (white blood cells)
Soman (10^{-5} M)	<5	2.3 ± 0.2
Paraoxon (10^{-4} M)	0	2.6 ± 0.3
BPNP (10^{-3} M)	66 ± 2	—
BPNP (10^{-4} M)	101 ± 3	66 ± 10
Physostigmin (10^{-5} M)	98 ± 2	106 ± 11
EDTA (10^{-3} M)	91 ± 4	—
40H-MB (10^{-3} M)	96 ± 2	97 ± 17

Values are mean values ± SD, N = 3–4 rats.
Inhibitors were preincubated at 37° for 30 min before incubation with 0.54 mM T-2 toxin.
Abbreviations: BPNP, bis-4-nitrophenyl phosphate; 40H-MB, 4-hydroxy mercurybenzoate.

Table 4. Hydrolysis of 0.54 mM T-2 toxin by white blood cells isolated from rat

Cell fraction	nmole HT-2 produced (mg protein·hr)	CarbE activity μmole 4NPB (mg protein·hr)
Monocytes	34 ± 9	2.7 ± 0.9
Lymphocytes	38 ± 5	1.4 ± 0.3
Granulocytes	49 ± 5	0.16 ± 0.04
Trombocytes	56 ± 2	1601 ± 222

The numbers are mean values ± SD, N = 3–4 animals.

that these cells possess carboxylesterase activity. This activity, however, did not correlate with T-2 toxin hydrolysis, indicating that 4-nitrophenylbutyrate is not a specific substrate for the isoenzyme responsible for T-2 hydrolysis, in agreement with a previous study at our laboratory on the heterogeneity of the carboxylesterases in liver [7].

DISCUSSION

Previous *in vitro* studies on metabolism of T-2 toxin have concentrated on liver preparations. The only report on screening of hydrolytic activity in organs other than liver is made by Ohta *et al.* [6]. In addition to the main metabolic organ which is the liver they found some activity in brain, kidney, spleen and intestine, but they did not find any detectable activity in serum or blood cells. However, because of the evidence for esteratic activity in blood cells stated earlier [12–14], the present study was designed to examine for possible trichothecene hydrolytic activities in blood cells.

In the present work, both human and rat blood cells were shown to convert T-2 toxin to polar and less toxic metabolites more easily excreted, thus indicating that the blood cells take part in the detoxification of T-2 toxin. With respect to the verification of trichothecene mycotoxins in blood samples drawn from animals or humans, it should be brought in mind that hydrolysis does occur even after sampling if not appropriately stored by immediate freezing or otherwise inactivating hydrolysing activity in blood samples.

We conclude that the metabolism of T-2 toxin by red and white blood cells is performed by carboxylesterase since it was completely blocked by the serine esterase inhibitors paraoxon and soman, and that physostigmin at 10^{-5} M did not inhibit the hydrolysis, thus excluding the cholinesterase. Furthermore, we could exclude arylesterase and phosphoryl phosphatase since it was not inhibited by EDTA or 4-hydroxy mercurybenzoate, respectively. The carboxylesterases in both red and white blood cells are characterized by being less sensitive to BPNP, in accordance with the finding in a previous study of T-2 toxin metabolism by rat liver carboxylesterase pI 5.4 [7]. The six different rat liver carboxylesterases have recently been identified by Mentlein *et al.* [21] as alloenzymic forms of ES-3, ES-4, ES-8/ES-10 and ES-15 according to the genetic nomenclature recommended by Van Zutphen [22]. Carboxylesterase pI 5.4 corresponding to pI 5.6 according to

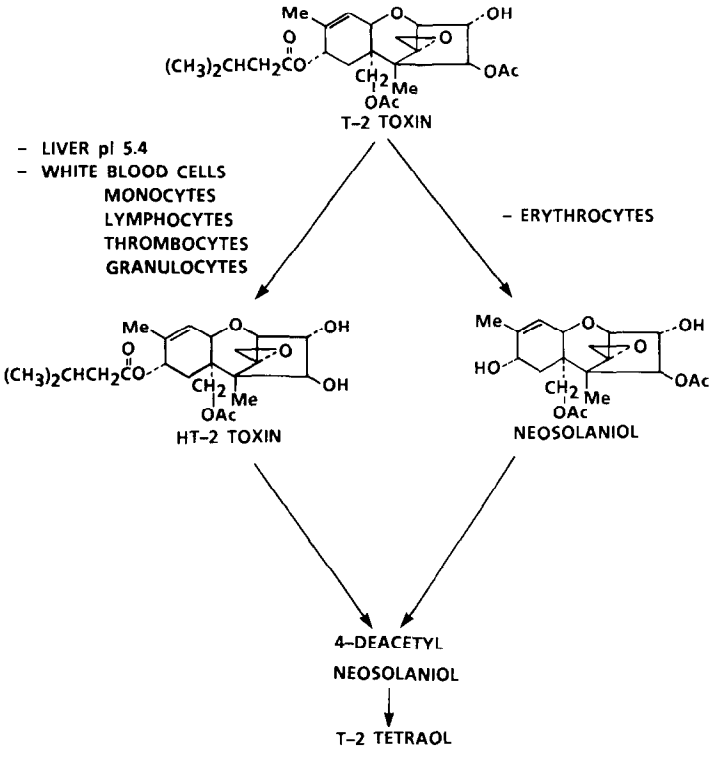


Fig. 2. Different pathways for the hydrolysis of T-2 toxin by carboxylesterases in liver and blood cells.

Mentlein *et al.* [23] was genetically defined as ES-3 [21]. According to the low sensitivity to BPNP for pI 5.4 enzyme hydrolysing T-2 toxin, which was also found for the carboxylesterases in red and white blood cells, and the fact that they all hydrolyse T-2 toxin, it may be suggested that the carboxylesterases present in the blood cells are of the same identity as liver carboxylesterase pI 5.4. On the other hand, small amounts of a nonspecific esterase defined as ES-13 with low affinity to BPNP have been demonstrated in liver and red blood cells with pI around 5.0 [24]. ES-13 can, however, not be responsible for the hydrolysis of T-2 toxin in liver because of their different pI-values, but it cannot be excluded as the enzyme responsible for the hydrolysis of T-2 toxin to neosolaniol in the red blood cells.

Of particular interest was the finding that neosolaniol is a major metabolite in addition to HT-2 toxin in blood. This metabolite is formed by hydrolysis of T-2 toxin at the isovaleryl group in C-8 position whereas HT-2 toxin is formed by hydrolysis of T-2 toxin at the acetyl group in C-4 position (Fig. 1). The C-4 acetyl residue has been found to be the preferential site for microsomal hydrolysis in liver and the substituents at C-3 and C-8 contribute to the selective enzymatic hydrolysis of the C-4 acetyl residue of trichothecenes [25]. Neosolaniol has been considered only as a trace or minor metabolite presumably formed by hydrolysis of T-2 toxin by dermal microflora [9, 10]. However, we provide evidence for erythrocytes as a specific site for enzymatic hydrolysis of T-2 toxin to neosolaniol, thus representing a metabolic pathway different from that of T-2 to HT-2 toxin, which is the pathway for T-2 toxin hydrolysis in liver and white blood cells. In fact, the hydrolytic activity in rat blood was completely separated into a HT-2 toxin producing pathway in white blood cells and another in red blood cells producing neosolaniol as primary metabolite. It was therefore concluded that the carboxylesterase in erythrocytes is different from carboxylesterase isoenzyme pI 5.4 in liver and the carboxylesterase present in the white blood cells. It may well be that the rigid structure of trichothecenes makes these an ideal substrate for characterizing certain groups of carboxylesterases. A summary of the two different pathways for hydrolysing T-2 toxin is shown in Fig. 2.

REFERENCES

1. Ueno Y, Sato N, Ishii K, Sakai K, Tsunoda H and Enomoto M, Biological and chemical detection of trichothecene mycotoxins of *Fusarium* species. *Appl Microbiol* **25**: 699–704, 1973.
2. Ueno Y, Mode of action of trichothecenes. *Pure Appl Chem.* **49**: 1737–1745, 1977.
3. Rosen RT and Rosen JD, Presence of four Fusarium mycotoxins and synthetic materials in "Yellow Rain". *Biomed Mass Spectrom* **9**: 443–450, 1982.
4. Mirocha CJ, Pawlosky RA, Chatterjee K, Watson S and Hayes W, Analysis for Fusarium toxins in various samples implicated in biological warfare in Southeast Asia. *J Assoc Off Anal Chem* **66**: 1485–1499, 1983.
5. Watson SA, Mirocha CJ and Hayes AW, Analysis for trichothecenes in samples from Southeast Asia associated with "Yellow Rain". *Fund Appl Toxicol* **4**: 700–717, 1984.
6. Ohta M, Ishii K and Ueno Y, Metabolism of trichothecene mycotoxins I: Microsomal deacetylation of T-2 toxin in animal tissues. *J Biochem (Japan)* **82**: 1591–1598, 1977.
7. Johnsen H, Odden E, Lie Ø, Johnsen BA and Fonnum F, Metabolism of T-2 toxin by rat liver carboxylesterase. *Biochem Pharmacol* **35**: 1469–1473, 1986.
8. Yoshizawa T, Swanson SP and Mirocha CJ, *In vitro* metabolism of T-2 toxin in rats. *Appl Environ Microbiol* **40**: 901–906, 1980.
9. Matsumoto H, Ito T and Ueno Y, Toxicological approaches to the metabolite of Fusaria XII. *Jpn J Exp Med* **48**: 393–399, 1978.
10. Corley RA, Swanson SP and Buck WB, Glucuronide conjugates of T-2 toxin and metabolites in swine bile and urine. *J Agric Food Chem* **33**: 1085–1089, 1985.
11. Yoshizawa T, Sakamoto T and Okamoto K, *In vitro* formation of 3′-hydroxy T-2 and 3′-hydroxy HT-2 toxins from T-2 toxin by liver homogenates from mice and monkeys. *Appl Environ Microbiol* **47**: 130–134, 1984.
12. Yam LT, Li CY and Crosby WH, Cytochemical identification of monocytes and granulocytes. *Am J Clin Pathol* **55**: 283–290, 1971.
13. Tashian RE, Multiple forms of esterases from human erythrocytes. *Proc Soc Exp Biol Med* **108**: 364–369, 1961.
14. von Deimling O and de Looze S, Human red cell butyrylesterase, and its homologies in thirteen other mammalian species. *Human Genetics* **63**: 241–246, 1983.
15. Bøyum A, Isolation of mononuclear cells and granulocytes from human blood. *Scand J Clin Lab Invest* **21**: Suppl. 97:77–89, 1968.
16. Bøyum A, Isolation of lymphocytes, granulocytes and macrophages. *Scand J Immunol* **5**, Suppl. 5:9–15, 1976.
17. Bøyum A, Isolation of human blood monocytes with Nycodenz, a new non-ionic iodinated gradient medium. *Scand J Immunol* **17**: 429–436, 1983.
18. Marton AV and Kalow W, Inhibitors of aromatic esterase in human serum. *Biochem Pharmacol* **3**: 149–154, 1960.
19. Augustinsson K-B, Multiple forms of esterase in vertebrate blood plasma. *Ann NY Acad Sci* **94**: 844–860, 1961.
20. Cohen JA and Warring MGPJ, Purification and properties of dialkylfluorophosphates. *Biochim Biophys Acta* **26**: 29–39, 1957.
21. Mentlein R, Ronai A, Robbi M, Heymann E and von Deimling O, Genetic identification of rat liver carboxylesterases isolated in different laboratories. *Biochim Biophys Acta* **913**: 27–38, 1987.
22. Van Zutphen LFM, Revision of the genetic nomenclature of esterase loci in the rat (Rattus Norvegicus). *Transplant Proc* **15**: 1687–1688, 1983.
23. Mentlein R, Heiland S and Heymann E, Simultaneous purification and comparative characterization of six serine hydrolases from rat liver microsomes. *Arch Biochem Biophys* **200**: 547–559, 1980.
24. Simon B, de Looze S, Ronai A and von Deimling O. Identification of rat liver carboxylesterase isozymes (EC 3.1.1.1) using polyacrylamide electrophoresis and isoelectric focusing. *Electrophoresis* **6**: 575–582, 1985.
25. Ohta M, Matsumoto H, Ishii K and Ueno Y, Metabolism of trichothecene mycotoxins II. Substrate specificity of microsomal deacetylation of trichothecenes. *J Biochem (Japan)* **84**: 697–706, 1978.