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Activity Determination, Kinetic Analyses and Isoenzyme Identification of Gamma Glutamyltransferase in Human Neutrophils

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Gamma-glutamyltransferase (GGT, EC 2.3.2.2) which hydrolyzes glutathione (GSH), is required for the maintenance of normal intracellular GSH concentration. GGT is a membrane enzyme present in leukocytes and platelets. Its activity has also been observed in human neutrophils. In this study, GGT was purified from Triton X-100 solubilized neutrophils and its kinetic parameters were determined. For kinetic analyses of transpeptidation reaction, γ-glutamyl *p*-nitroanilide was used as the substrate and glycylglycine as the acceptor. Apparent K_m values were determined as 1.8 mM for γ-glutamyl *p*-nitroanilide and 16.9 mM for glycylglycine. The optimum pH of GGT activity was 8.2 and the optimum temperature was 37ºC. It had thermal stability with 58% relative activity at 56ºC for 30 min incubation. L-serine, in the presence of borate, was detected as the competetive inhibitor. Bromcresol green inhibited neutrophil GGT activity as a noncompetetive inhibitor. The neutrophils seem to contain only the isoenzyme that is present in platelets. We characterized the kinetic properties and compared the type of the isoenzyme of neutrophil GGT with platelet GGT via polyacrylamide gel electrophoresis (PAGE) under a standart set of conditions.

Keywords: Gamma-glutamyltransferase, Isoenzyme, Kinetics, Neutrophils

Introduction

The ectoenzyme gamma-glutamyltransferase (GGT, 2.3.2.2) is one of the key enzymes in the gamma glutamyl cycle (Meister and Anderson, 1983) that catalyses the hydrolysis of gamma-glutamyl compounds and transfer of amino acids or peptides. Gamma-glutamyl cycle involving GGT is generally thought to be the major pathway by which cells utilize extracellular GSH for de novo synthesis of intracellular GSH (Griffith *et al*., 1978). GGT was reported to be involved in the regulation of the level of glutathione (Meister, 1973), the metabolism of endogenous compounds like prostaglandins and the conversion of leukotriene C_4 to leukotriene D_4 (Ahorony, 1984; Pace-Asciak *et al*., 1986). Recently, it has been reported that GGT cleavage of GSH and subsequent recapture of cysteine and cystine allow cells to maintain low levels of cellular reactive oxygen species (ROS) and to avoid apoptosis induced by oxidative stress (Karp *et al*., 2001).

GGT has been demonstrated in various human and animal organs, including kidneys, liver and spleen. It is also present in various body fluids, such as saliva, serum, bile and urine (Nemesanzky and Lott, 1985; Sener, 1997). GGT has been detected both in blood and bone marrow cells by using cytochemical methods (Szmigielski *et al*., 1965). It has been demonstrated at the ultrastructural level on the surface membrane and membranes of endoplasmic reticulum and golgi apparatus of human lymphoid cells (Marathe *et al*., 1980). Girino et al. (1985) have demonstrated the existence of the enzyme in almost all normal leukocytes and in platelets at cytochemical level. In another study, it has been reported that GGT could serve as a differentiation marker in the study of granulocytic and lymphocytic cell lineages (Khalaf and Hayhoe, 1987).

GGT activity has also been observed in populations of non-stimulated mononuclear cells of human peripheral blood. The highest activities of GGT have been found in monocytes. Different activities have been observed in CD4 positive and CD8 positive T lymphocytes. It has been demonstrated that these differences might reflect different biochemical capacities of these cell types, related to their distinct functions in the immune system (Grisk *et al*., 1993). Recently It has been shown that resting peripheral blood T cells express a higher level of GGT than native T cells (Karp *et al*., 1999).

In our previous study, we both investigated the kinetic properties of GGT in platelets and demonstrated that there was

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only one GGT isoenzyme in human platelets (Yardimci *et al*., 1995; Sener and Yardimci, 2000).

In the present work, we have investigated the catalytic activity, kinetics properties, inhibitor effects, optimum pH, optimum temperature and thermal stability of human neutrophil GGT. At the same time, neutrophil GGT isoenzyme was compared with platelet GGT isoenzyme on PAGE.

Materials and Methods

Materials Gamma-glutamyl p-nitroanilide, glycylglycine, Triton X-100, Con A Sepharose 4B (Ricinus Communis I), methyl α-D-mannopyranoside, naphtylethylene diamine, dextrane, L-serine, bromcresol green and ficoll hypaque were purchased from Sigma Chemical Co. (St Louis, USA). Other chemicals were of reagent grade from Merck (Darmstadt, Germany).

Isolation of neutrophils Blood was drawn from normal human volunteers and anticoagulated with 0.077 M EDTA (1 : 9 ratio). Neutrophils were isolated from plasma containing leukocytes and platelets by dextran sedimentation followed by Ficoll hypaque gradient centrifugation (Boyum, 1968). The residual red blood cells were lysed by hypotonic saline at 4°C and the purified neutrophils were resuspended in phosphate buffered saline (PBS, 125 mM NaCl, 8 mM NaH_2PO_4, 5 mM KCl, 5 mM glucose, pH 7.4) and stored on ice in a Ca^{2+} and Mg^{2+} free medium. The final preparation contained more than 90%-95% neutrophils. The isolated neutrophils were washed three times in PBS and concentrated to 5×10^6 cells *per* ml in PBS containing 1% Triton X-100. Then, the cells were left standing for 5 h, with gently stirred and centrifuged at 10.000 rpm for 15 min at 4°C to give a clear supernatant.

Gamma-glutamyltransferase assay The GGT activity was determined by the hydrolysis of g-glutamyl p-nitroanilide in the presence of the acceptor glycylglycine (Szasz, 1969). Standard assay included final reagent concentrations 4 mM of γ-glutamyl *p*-nitroanilide, 40 mM of glycylglycine and 185 mM of Tris-HCl, pH 8.2. The rate of p-nitroaniline formation was measured at 405 nm by using spectrophotometer. The results were expressed as pkat *per* 10^6 neutrophils using milimolar absorptivity of 9.900. One katal of activity is defined as amount of enzyme that will catalyze the formation of one mole of p-nitroaniline per second under the conditions of the assay procedure. Serum GGT activity was expressed as U/L. One Unit of enzyme represents the amount of enzyme that catalyzes the release of 1mmol of nitroaniline/min. The assay was used in all steps of the isolation of neutrophil GGT and kinetic studies. Protein levels were determined according to the method of Bradford (1976) using bovine serum albumin as the standard.

Purification of neutrophil GGT The method of Vesely *et al*. (1985) was used with some modifications. Triton X-100 solubilized neutrophil fractions from nine subjects were first pooled and then precipitated with ammonium sulphate (45-90% saturation). The precipitated proteins were dialyzed and chromatographed on a Sephadex G-200 column. Fractions containing GGT activities of the Sephadex G-200 were pooled and incubated for 3 h at 37°C with Con A Sepharose 4B. Con A Sepharose 4B was packed into a column (0.9 × 5 cm) and washed with 10 mM Tris HCl buffer containing 0.2% Triton X-100, 0.5 M NaCl, 10 mM $MgCl_2$ and 10 mM $CaCl_2$ until no protein could be eluted. Then, the bound protein was eluted with the same buffer containing 0.3 M methyl α-D-mannopyranoside.

Kinetic studies of neutrophils Transpeptidation reaction of GGT examined between two substrates. The K_m and V_{max} estimated at fixed set of cosubstrate concentrations is not the true values but rather apparent values. Therefore these parameters are referred as apparent K_m, apparent V_{max}.

The GGT activity was measured at various concentrations (1-10 mM) of the substrate γ-glutamyl p-nitroanilide and 40 mM glycylglycine as the acceptor at 37°C at pH 8.2 for transpeptidation reaction. All of the experiments were performed in duplicates. Apparent K_m and apparent V_{max} values were determined from Lineweaver Burk plots for γ-glutamyl *p*-nitroanilide. Concentration of the acceptor glycylglycine for determination of apparent K_m and apparent V_{max} values varied between 10-100 mM, and γ-glutamyl *p*-nitroanilide concentration was 4 mM at 37°C at pH 8.2.

Determination of optimum temperature and optimum pH The neutrophil GGT was incubated at 56°C at different time periods (5-30 min) keeping all other variables constant for the thermal stability determination. For optimum temperature detection, the GGT activities were determined after incubation for 1 min and 10 min at different temperatures (25-56ºC). The reaction mixture contained 4 mmol of γ-glutamyl p-nitroanilide *per* L and 40 mM of glycylglycine *per* L.

Enzyme activity was followed with respect to pH variations using Tris-HCl buffer (pH 5-7) and phosphate buffer (pH 8-9) containing 4 mmol of γ-glutamyl *p*-nitroanilide *per* L and 40 mmol of glycylglycine *per* L.

Inhibition studies The GGT inhibition with L-serine-borate mixture and with bromcresol green was studied by using Tris-HCl buffer containing various concentrations of γ-glutamyl *p*-nitroanilide (1-10 mM) and 40 mmol of glycylglycine *per* L. L-serine (2.5 mM) and borate (10 mM) were added to the reaction mixtures. Bromcresol green concentration was 0.01 mM. The GGT activities were assayed after 1 min incubation with the inhibitors.

Detecting the enzyme activities on gels Neutrophil GGT, purified by Con A Sepharose 4B column chromatograpy, was electrophoresed in polyacrylamide gels (7.5%) according to the method of Davis (1964) with Tris-glycine buffer (pH 8.3). The GGT staining was performed as described by Selveraj and Balasubramanian (1982). Gels, soon after polyacrylamide gel electrophoresis (PAGE), were immersed in a staining solution containing 185 mM Tris-HCl, 4 mM γ-glutamyl *p*-nitroanilide, 80 mM glycylglycine, 0.1% sodium nitrite and 0.4% naphtyl ethylenediamine dihydrochloride (pH 8.2) and incubated at 37°C for 30 min. The gels were then washed with 10 mM Tris-HCl (pH 8.9) and transferred to cold 0.38 mM trichloroacetic acid. Within 10 min, a pink band appeared on the gels in the region of GGT. Protein staining was accomplished with Commasie Brillant Blue R-250.

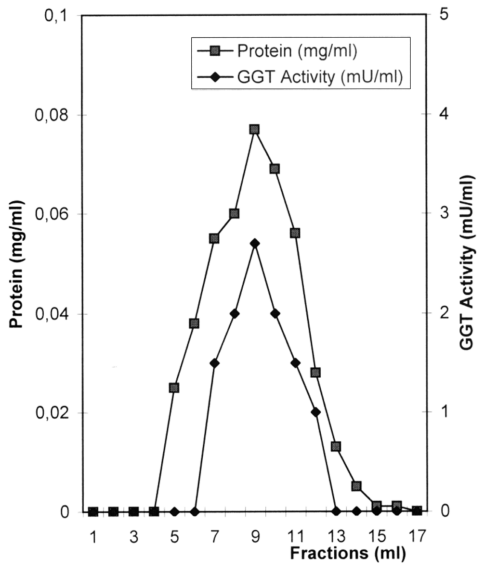

Fig. 1. Elution profile of Con A sepharose 4B column chromatography (0.9 × 5 cm) in 10 mM Tris HCl buffer (pH 8.2) containing 0.2% Triton X-100, 0.5 M NaCl, 10 mM $MgCl_2$, 10 mM $CaCl_2$ and 0.3 M methyl α-D-mannopyranoside.

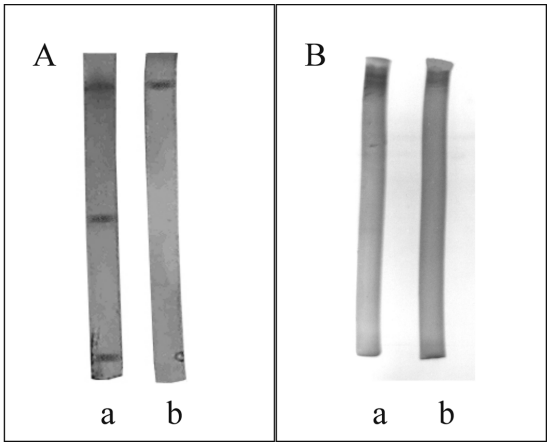

Fig. 2. (A) The results of PAGE with protein stainning (a, Triton X-100 solubilized proteins of human neutrophils, b, Purified human neutrophile GGT) (B) Gels of GGT specific staining (a, Human platelet GGT, b, Human neutrophile GGT).

Results

The GGT activities of Triton X-100 solubilized neutrophils from nine healthy subjects between 20-30 years of age were determined. The median neutrophil GGT activity and the serum GGT activity were 6.2 ± 1.3 pkat/10^6 neutrophils and 13 ± 3 U/L respectively.

Solubilized fractions with Triton X-100 of nine subjects (their GGT activities varying between 5.0-8.0 pkat/10^6 neutrophils) were pooled and used for purification. The human neutrophil GGT was purified by lectin affinity chromatography on Con A Sepharose 4B. The specific activity of purified GGT was 31.3 mU/mg protein. In Fig. 1, the Con A Sepharose 4B column chromatography elution profile is shown. The fractions with high specific activities were pooled. When PAGE was applied to the purified GGT from Triton X-100 solubilized neutrophiles, a single protein band was observed. It was shown that there was only one GGT isoenzyme in neutrophils with GGT staining on the PAGE. It was observed that the electrophilic mobility of this protein had the same electrophilic mobility with the platelet GGT isoenzyme (Fig. 2).

The Lineweaver Burk plots of the γ-glutamyl *p*-nitroanilide concentration dependency of human neutrophil GGT of normal cases are seen in Fig. 3. The apparent K_m and apparent V_{max} values were calculated as 1.8 mM and 12.1 pkat/10^6 neutrophils. For acceptor glycylglycine, apparent K_m and

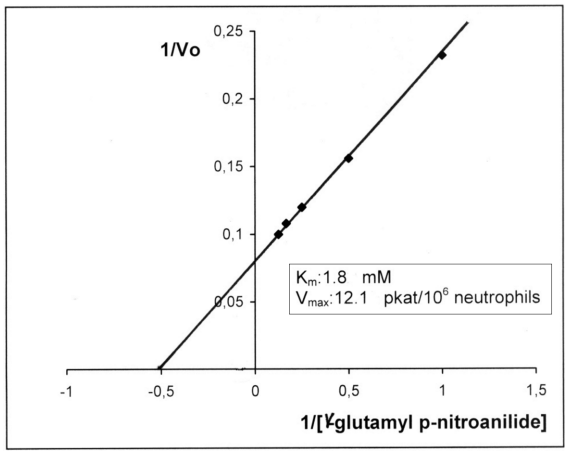

Fig. 3. Lineweaver Burk plot for human neutrophile GGT with γ-glutamyl *p*-nitroanilide as substrate. The reaction mixture contained 185 mM Tris-HCl buffer (pH: 8.2), 40 mM glcylglycine and varying concentrations of γ-glutamyl *p*-nitroanilide.

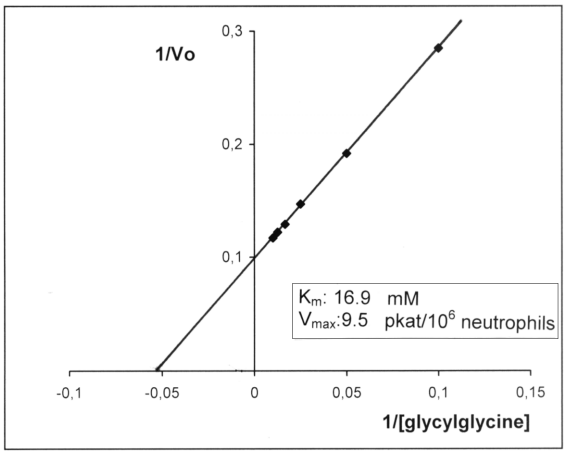

Fig. 4. Lineweaver Burk plot for human neutrophile GGT with glcylglycine as acceptor. The reaction mixture contained 185 mM Tris-HCl buffer (pH: 8.2), 4 mM g-glutamyl *p*-nitroanilide and varying concentrations of glcylglycine.

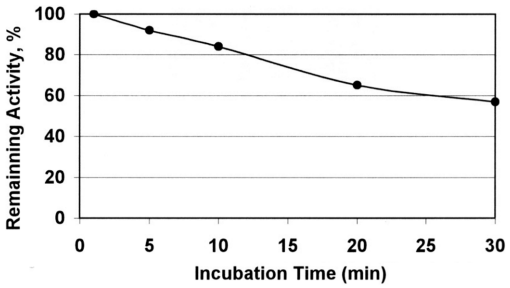

Fig. 5. Thermal stability of human neutrophile GGT activity at 56°C.

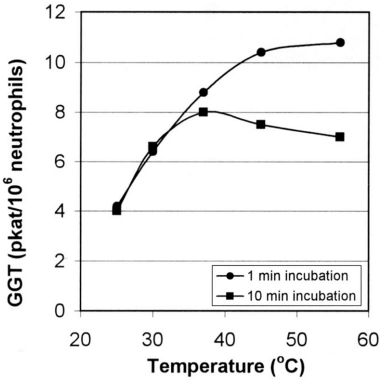

Fig. 6. Effect of temperature on neutrophile GGT activity.

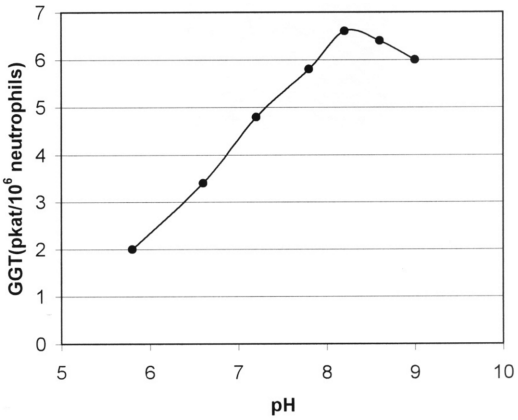

Fig. 7. Effect of pH on neutrophile GGT activity.

apparent V_{max} values were 16.9 mM and 9.5 pkat/10^6 neutrophils from Lineweaver Burk Plots (Fig. 4).

When the enzyme was heated at 56°C for varying periods, the neutrophil GGT, like platelet GGT, had high thermal stability. 58% of relative activity remained after 30 min incubation at 56°C (Fig. 5). The optimum temperature was determined to be 37°C (Fig. 6), and the optimum pH was 8.2 (Fig. 7), when 4 mM γ-glutamyl p-nitroanilide was used as the substrate and 40 mM glycylglycine as the acceptor.

In the presence of borate (10 mM), L-serine (2.5 mM) competitively inhibited 32% of the neutrophile GGT activity at 4 mM concentration of γ-glutamyl p-nitroanilide (Fig. 8).

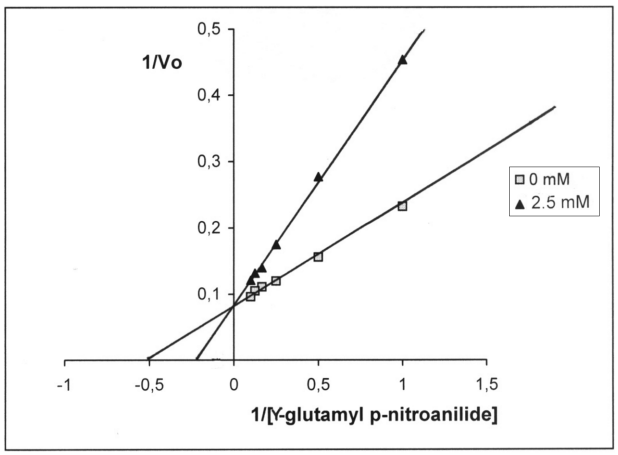

Fig. 8. Lineweaver Burk plot of L-serine (2.5 mM) inhibition on the activity of neutrophile GGT at 37°C in the presence of 10 mmol/L borate.

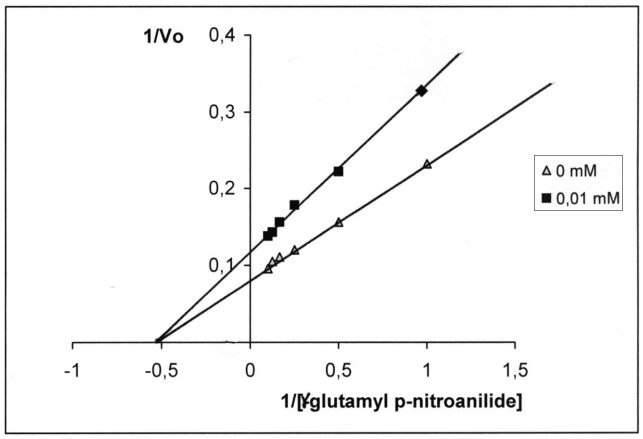

Fig. 9. Lineweaver Burk plot of bromcresol green (0.01 mM) inhibition on the activity of neutrophile GGT at 37°C.

Inhibition of enzyme activity by Bromcresol green (0.01 mM) caused 31% inhibition at 4 mM concentration of γ-glutamyl *p*-nitroanilide. This inhibition was noncompetitive inhibition as determined from Lineweaver Burk Plots (Fig. 9).

Discussion

GGT is a heterodimeric glycoprotein anchored to the outside surface of the plasma membrane of both animal and plant cells (Abbott *et al*., 1982). GGT can be solubilized by using detergents such as TritonX-100 and deoxycholate or by treatment with proteinases (e.g. papain). Detergents solubilized the entire molecule, whereas proteinases cleave the hydrophilic active site containing the domain from the membrane bound anchor (Nemesanzky and Lott, 1985). We also observed that papain solubilized GGT had a higher mobility than Triton X-100 solubilized GGT. This difference are related to the amino acid chain length (Sener and

Yardimci, 2000).

Species and tissue differences were described in terms of the GGT affinity to Con A lectin (Goldstein *et al*., 1965; Shaw *et al*., 1978; Dvorakova *et al*., 1992). It is reported that differences in Con A binding properties cannot be related structures (Dvorakova *et al*., 1992b). However, after neuraminidase treatment, reduction in Con A binding, dependent on sialic acid content, has been reported (Delanghe *et al.*, 1987). In our previous work, the incubation of platelet GGT with Con A sepharose resulted in the binding of 73% of the activity to gel (Sener and Yardimci, 2000).

The GGT isoenzyme bands are characterized by their relationship with the major serum protein fractions. Normal serum GGT has been separeted in two fractions, namely α_1-globulin and α_2-globulin by cellulose acetate electrophoresis (Burlina, 1978). Increased serum levels and isoenzyme patterns of GGT has been noted in some pathological conditions such as liver, biliary tract disorders and pancreatic diseases. Four GGT isoenzymes that were named as GGT1, GGT2, GGT3 and GGT4 were determined in albumin, α_1-globulin, α_2-globulin and β-globulin fractions respectively (Burlina, 1978, Nemesanzky and Lott, 1985). The electrophoretic heterogenity of the GGT isoenzymes in serum was reported to be due to post-translational modification (Nemesanzky and Lott, 1985). However, Pawlak *et al*. (1988) showed that at least four genes containing GGT sequences were present in the human genome. These are located at band q11 on chromosome 22 (Morris *et al*., 1993; Collins *et al*., 1997). Recently, It has been reported that the human GGT was encoded by a multigene family including at least seven genes. These genes have not fully characterized. Only one of genes (gene I) was found functional and produced multiple transcripts that were translated into complete and functional proteins. In human, This gene codes least three 5' altering mRNAs (Chikhi *et al*., 1999). It has been suggested that human GGT gene employs regulatory sequences and alternative splicing (Visvikis *et al*., 2001). Therefore, isoenzymes of GGT may be related tissue specific expression. In our previous work, we showed that human platelets contained only one isoenzyme (Sener and Yardimci, 2000). Gürdöl *et al*. (1995) also reported that human platelets contained one isoenzyme, GGT 4.

In the study with serum GGT, it was reported that for γ-glutamyl *p*-nitroanilide and glycyl glycine, the K_m values were 1.9 mM and 12.0 mM respectively (Rosalki and Tarlow, 1974). We have shown that for γ-glutamyl p-nitroanilide and glycyl glycine, in the platelets of normal subjects, the K_m values were calculated as 2.1 mM and 15.9 mM respectively (Yardimci *et al*, 1995). It was reported in the literature that in human brain capillaries, the partially purified GGT had optimum pH between 8.2 and 9 (Vesely *et al*., 1985). The K_m value of human liver GGT, when γ-glutamyl *p*-nitroanilide was used as the substrate, was reported as 1.4 mM, and for kidney it was 1.2 mM. For glycylglycine, the K_m value was 10.6 mM for the liver and 10.7 the mM for kidneys (Shaw *et al*., 1978).

L-serine against borate and bromcresol green are widely used inhibitors of GGT in kinetic studies. L-serine inhibits GGT in the presence of borate by interacting with the γ-glutamyl binding site of the enzyme (Tate and Meister, 1978; Vesely *et al*., 1985). Purified rat deciduoma had an optimum pH of 8.2 and a K_m value of 1 mM for γ-glutamyl *p*-nitroanilide and 7.6 mM for glycylglycine. L-serine, in presence borate, inhibited GGT competetively, K_i being 22 μM (Taracand, 1984).

Brom cresol green inhibited neutrophil GGT noncompotetively whereas in presence of borate, L-serine inhibited competetively. The neutrophil GGT kinetics showed similarities with platelet GGT and some differences from K_m value of γ-glutamyl *p*-nitroanilide in other tissues. We think that the differences are related to different isoenzyme.

Gamma-glutamyltransferase was also found as a membrane enzyme in immune cells using immunocytochemical and biochemical methods (Khalaf and Hayhoe, 1987). Investigations showed activity in almost all bone marrow and blood cells, but it was weaker in these cells. The GGT activities were found 7.3-20.0 pkat/10^6 cells in monocytes (Grisk *et al*., 1993).

Primary human memory T cells express higher level of GGT than native T cells. It has been suggested that GGT might play an important role in the regulation of lymphocytes which are at a particular developmental stage (Karp *et al*.,1999). At the same time, GGT expression, highly sensitive to oxidative stress, is a part of the cell antioxidant defence mechanisms (Karp *et al*., 2001).

GGT activity is increased in a number of primary and metastatic tumors (Dominici *et al*., 2003). It has been shown that hepatocellular carcinoma can be expressed hepatoma specific GGT and secreted GGT in to circulating blood (Yao *et al*., 1998). Beltran-Martinez and Correa-Chacon (2003) suggested that GGT was a useful marker of metastatic disease in patients with renal cell carcinoma. GGT activity also changes several types of lekuemia (Tager *et al*., 1995). Novogrodsky *et al*. (1976) indicated that GGT is lymphoid cell surface marker reflecting differantiation in normal and malignant cells. The GGT activity of blood lymphoid cells of normals and Acute Lymphoblastic Leukemia (ALL) were determined as 2.3 ± 0.3 U/mg protein and 1.1 ± 0.2 U/mg protein respectively (Russo *et al.,* 1987). By contrast, it was reported that GGT levels increased in acute myeloid leukemia, chronic myeloid leukemia and high grade non Hodgkins lymphoma (Antczak *et al*., 2001).

In our studies, the kinetics of GGT is described in human neutrophil. The differences in our results, when compared with other tissues, might be due to the presence of different isoenzymes. The optimum pH and temperature values of neutrophils were similar to other cells, as reported in the literature. We have showed that human neutrophils contains a GGT isoenzyme similar to the platelet isoenzyme. These studies may contribute to the use of GGT in diagnosis and

prognosis of various haematological disorders and to understand the function of GGT in leukocytes.

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