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Biochemical Pharmacology, Vol. 29, pp. 1859–1861.

0006-2952/80/0615-1859 $02.00/0

Evidence for the external location of alkaline phosphatase activity on the surface of Sarcoma 180 cells resistant to 6-thioguanine

(*Received* 16 *November* 1979; *accepted* 29 *December* 1979)

The 6-thiopurines (i.e. 6-mercaptopurine and 6-thioguanine) are effective agents in the treatment of acute leukemia in man. One of the factors which limits the therapeutic usefulness of these antimetabolites is the acquisition of insensitivity by previously susceptible leukemic cells. To exert antineoplastic activity, conversion of these agents to the nucleotide level is mandatory, and most transplanted tumours achieve resistance by deletion or alteration of hypoxanthine-guanine phosphoribosyltransferase (HGPRT) activity, the enzyme system involved in the formation of 6-thiopurine 5'-phosphate [1]. Acute lymphocytic leukemic cells of man, however, do not appear to decrease HGPRT activity as the mechanism of acquired resistance to these purine antimetabolites [2–5]. Evidence is available in a subline of the murine ascitic neoplasm Sarcoma 180 resistant to the 6-thiopurines (S180/TG) which suggests that insensitivity is the result of an increased rate of degradation of 6-thiopurine 5'-phosphate by alkaline phosphatase [6, 7]. Furthermore, leukocytes of acute lymphocytic leukemia patients demonstrated in most instances an increase in particulate-bound alkaline phosphatase activity that corresponded to acquired clinical insensitivity to the 6-thiopurines [4]. At least two particulate-bound alkaline phosphatase activities occur in S180/TG cells which are distinct in several characteristics and these have been purified to homogeneity [7–9]. The levels of these enzymes, which appear to be responsible for the degradation of 6-thiopurine mononucleotide in S180/TG cells, are elevated about 100-fold in the resistant tumor [7–9]. The cellular localization of these two isoenzymes has not been studied, and this communication is concerned with our initial observations on the location of these enzymes in S180/TG cells. Although intracellular phosphatase activity would appear to be required to express resistance to the 6-thiopurines, the findings presented in this report suggest that a significant portion of the cellular alkaline phosphatase catalytic activity resides on the external surface of the cell membrane.

Tumor cells were maintained in female CD-1 mice (Charles River Breeding Laboratories, Wilmington, MA) by the weekly intraperitoneal inoculation of $1–2 \times 10^6$ cells/mouse. The activity of alkaline phosphatase was measured spectrophotometrically by following the increase in absorbance at 410 nm in phosphate-buffered saline (PBS; 0.137 M NaCl, 0.003 M KCl and 0.008 M Na_2HPO_4-KH_2PO_4) containing 1.0 mM *p*-nitrophenylphosphate (PNPP) at 37° as described previously [7]. The assay of enzyme activity of intact cells employed incubation (2×10^6 cells/ml) in the above medium for varying periods of time, followed by measurement of the absorbance of the medium after removal of cells by centrifugation for 1 min at 1600 *g*.

The rate of hydrolysis of PNPP to *p*-nitrophenol by intact S180/TG cells was about 2.7 times faster than that produced by the parent drug-sensitive Sarcoma 180 cells (S180) at pH 7.4 (Fig. 1). Since PNPP is negatively charged, it was assumed that this material was taken up poorly by cells; *p*-nitrophenol, however, might be expected to readily permeate cells. Thus, measurement of this material in the medium should underestimate somewhat the quantity of this product formed from intact cells. The possibility that S180/TG cells secreted greater amounts of hydrolytic enzyme activity into the medium than S180 cells was eliminated by the observation that phosphohydrolase activity was minimal in medium freed of cells after incubation of each cell line for up to 45 min (Fig. 2); this finding also indicated that hydrolytic activity was largely associated with

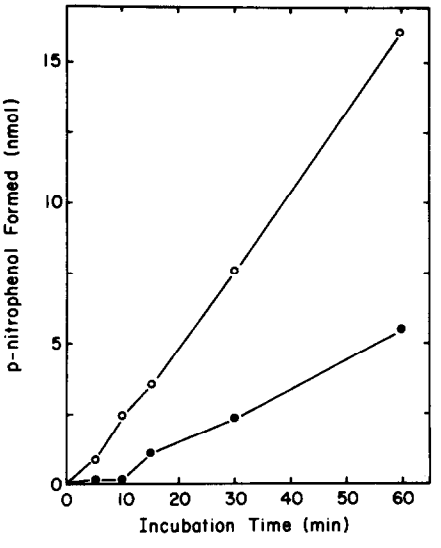

Fig. 1. Hydrolysis of PNPP by intact S180 and S180/TG cells. Cells (2×10^6/ml) were incubated with 1 mM PNPP at 37° in PBS (pH 7.4). At various times thereafter, the absorbance at 410 nm of the supernatant solution was measured, using a supernatant fraction of an incubation mixture without PNPP as the blank. The molar absorbance of *p*-nitrophenol employed was $1.7 \cdot 10^4$ moles $\cdot l^{-1} \cdot cm^{-1}$. Key: phosphohydrolase activity of S180 (●——●) and of S180/TG (○——○).

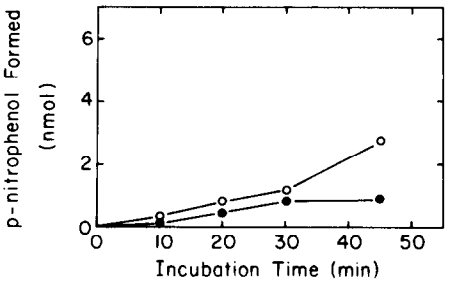

Fig. 2. Hydrolysis of PNPP by medium after incubation of 2×10^6 S180 or S180/TG cells per ml in PBS (pH 7.4) for various periods of time at 37°. The catalytic activity of the resulting supernatant solution (i.e. the medium) was assayed by incubation in the presence of PNPP for 30 min at 37°; see legend of Fig. 1 for assay details. Key: phosphohydrolase activity of the medium following incubation with S180 (●——●) and S180/TG (○——○).

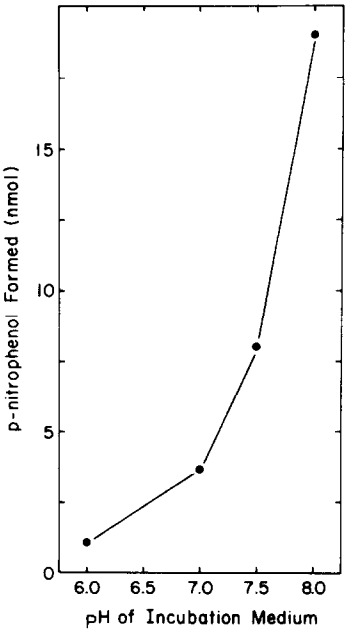

Fig 3. Effect of pH on the hydrolysis of PNPP by S180/TG cells. Assays were performed in PBS (pH 6.0 to 8.0) by a 30-min incubation of cells in the presence of PNPP at 37°. See legend of Fig. 1 for assay details.

intact cells. Variation of the pH of the incubation medium from pH 6.0 to 8.0 with S180/TG cells showed about 5-fold more hydrolytic activity at pH 8.0 than at pH 7.0, supporting the concept that alkaline phosphatase activity was responsible for the hydrolysis of PNPP (Fig. 3). These findings imply that a significant amount of the catalytic activity of at least one of the alkaline phosphatase isoenzymes of S180/TG is expressed on the external surface of the cells. We have attempted to obtain further evidence for the surface location of alkaline phosphatase by iodination of the plasma membrane of S180/TG cells by the lactoperoxidase method as described by Phillips and Morrison [10], followed by purification and separation on polyacrylamide gels of alkaline phosphatase isoenzymes [7]. These experiments showed that neither band of the two major alkaline phosphatase activities present in S180/TG cells corresponded to the peaks of radioactivity present on these gels (data not shown). The failure to label the external surface alkaline phosphatase activity by iodination presumably was due to the absence of available tyrosine residues in that portion of enzyme present on the external surface of the S180/TG cell membrane.

Several studies have been conducted on the cellular localization of alkaline phosphatase activity in other systems; these investigations have demonstrated that enzymatic activity was located predominantly in the peripheral plasma membrane [11–13]. Electron microscopic studies have also provided evidence in two clones of HeLa that catalytic alkaline phosphatase activity is expressed on the external surface of these cells [14]. The function of alkaline phosphatase activity is obscure, and hydrolytic, transphosphorylation and pyrophosphatase actions have been suggested for this enzyme [15]. It is conceivable that the phosphohydrolytic activity present on the external surface of cells may be involved in scavenging phosphorylated compounds present in the environment of the cells. The physiologic source of such materials is obscure, but may include nucleotides, phospholipids and phosphorylated sugars which occur in plasma [16].

Should human acute lymphocytic leukemia cells resistant to the 6-thiopurines express a portion of their elevated particulate alkaline phosphatase activity on the external surface of the cell membrane in a manner analogous to S180/TG, it is possible that therapeutic advantage of the acquisition of resistance to these agents might be attained by employing phosphorylated derivatives of cytotoxic agents as prodrugs. Such agents are negatively charged, and consequently poorly transported into most cells; however, preferential dephosphorylation by 6-thiopurine-resistant leukemia cells would lead to high concentrations of a transportable form of chemotherapeutic agent in the environment of these cells, thereby exploiting the property of acquired insensitivity through this biochemical mechanism. A useful agent of this kind might be one that would (a) become more lipophilic and transportable by passive diffusion after dephosphorylation, and (b) generate a reactive form following removal of phosphate, thereby creating alkylations in thiopurine-resistant cells. Dephosphorylation of 5′-nucleotides by humans appears to be relatively poor, presumably due to low levels of kidney and blood phosphohydrolase activity [17, 18]. Thus, it is possible that, in man, phosphorylated prodrugs of cytotoxic agents might have a sufficiently long blood half-life to be of significance in the treatment of patients with acute lymphocytic leukemia resistant to the 6-thiopurines through increased alkaline phosphatase activity.

Acknowledgements—This investigation was supported by Grant DRG-205-FT from the Damon Runyon–Walter Winchell Cancer Fund, and U.S. Public Health Service Grants CA-02817 and CA-16359 from the National Cancer Institute.

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Biochemical Pharmacology, Vol. 29, pp. 1861–1863.

0006-2952/80/0615-1861 $02.00/0

Cocaine: effect of *in vivo* administration on synaptosomal uptake of norepinephrine

(*Received* 24 *September* 1979; *accepted* 11 *December* 1979)

Ever since reports of coca leaf chewing filtered back to Europe in the wake of the Spanish conquest of the Andes, interest in cocaine has waxed and waned in sympathy with social currents which encouraged or discouraged its use. Consequently, with the increase in illicit cocaine traffic which has taken place since the mid-1960's [1], research interest in cocaine has been rejuvenated. Recent investigations have confirmed that cocaine is a central nervous system stimulant. As currently reviewed [2], in humans cocaine significantly reduces total and rapid eye movement (REM) sleep. It produces euphoria, anorexia, perceptual and affective changes at low doses, and paranoid psychoses at higher doses. In experimental animals, cocaine increases locomotor activity and body temperature, and elicits stereotyped behavior and turning toward the lesioned side in animals with unilateral lesions of the nigro-striatal dopaminergic pathway. It also reduces food intake, induces a desynchronized electroencephalogram (EEG), and increases multiple unit activity in the reticular formation.

It has been theorized that the CNS excitation may result from inhibition of reuptake of endogenously produced catecholamines, particularly norepinephrine (NE) [3], though an effect of *in vivo* cocaine on catecholamine uptake in brain tissues has not been demonstrated previously. Reuptake is considered to be the primary mechanism by which catecholamines are inactivated [4]. Inhibition of this process would permit neurotransmitter to linger within the synaptic cleft and prolong any post-synaptic actions of released neurotransmitter. Reuptake is mediated by an energy dependent, high affinity transport system (uptake 1) located at the axonal membrane of neurons [5] and is highly sodium-ion dependent. In the peripheral nervous system, cocaine has been shown to be a potent inhibitor of uptake 1 but has little effect on uptake 2, a high capacity, low affinity system with many differing characteristics from uptake 1 [6, 7].

The action of cocaine is on the neuronal membrane, while it is less effective in inhibiting uptake by granular (vesicular) membrane [8]. Moreover, the uptake of NE by sympathetic nerve cell bodies appears to be considerably less sensitive to cocaine inhibition than the uptake of this transmitter by nerve endings [9, 10]. The inhibition is competitive [11, 12], that is, cocaine vies with NE for uptake receptor sites.

Though cocaine inhibition of NE uptake has been demonstrated both *in vitro* and *in vivo* in a large number of peripheral tissues [2], unfortunately less data are available for the central nervous system. Moreover, the results have been contradictory. Decreased NE uptake has been reported using *in vitro* incubations with mouse synaptosomes [13], brain slices of cats [14], mice [15] and rats [11], chopped rat brains [16] and rat pineals [17]. But the rare *in vivo* studies have not demonstrated decreased NE uptake, though a reduction in [^{3}H]NE retention after its intraventricular injection into cocaine-treated rats has been reported [18]. Intraventricular injections of tritiated NE showed no decrease in NE uptake in animals receiving 15 mg/kg of cocaine compared with controls [19], and brain slices of mice treated with 40 mg/kg of cocaine took up tritiated NE to the same extent as saline-treated animals [15]. This suggested that cocaine might not have the same action in the brain as it does in the peripheral nervous system. However, the numerous problems in methods, including their sensitivity, coupled with the uncertainties in the interpretation of the results of *in vivo* uptake studies carried out in intact brain preparations, as outlined by Maxwell *et al.* [12], mitigate against the use of these techniques in characterizing the CNS actions of cocaine. We chose to surmount the CNS *in vivo* problems by performing an *in vivo* cocaine study on nerve endings that would subsequently be isolated from supporting glia and the blood–brain and liquor–brain barriers (synaptosomes). This would permit us to see if they would react to endogenously bound cocaine as they do to the drug *in vitro*. The synaptosomal preparation also permitted us to examine such discrete details of NE uptake as K_m and V_{max} (Michaelis–Menten analysis).

Adult male Sprague–Dawley rats, each weighing 350 g.