ABSTRACT:
Detoxification of cyclophosphamide is effected, in part, by hepatic class 1 aldehyde dehydrogenase (ALDH-1)-catalyzed oxidation of aldophosphamide, a pivotal aldehyde intermediate, to the nontoxic metabolite, carboxyphosphamide. This enzyme is found in erythrocytes as well. Detoxification of aldophosphamide may also be effected by enzymes, viz. certain aldo-keto reductases, that catalyze the reduction of aldophosphamide to alcophabosphamide. Such enzymes are also found in erythrocytes. Not known at the onset of this investigation was whether the contribution of erythrocyte ALDH-1 and/or aldo-keto reductases to the overall systemic detoxification of circulating aldophosphamide is significant. Thus, NAD(P)-linked oxidation and NADPH-linked reduction of aldophosphamide catalyzed by relevant erythrocyte enzymes were quantified. ALDH-1-catalyzed oxidation of aldophosphamide (160 μM) to carboxyphosphamide occurred at a mean (± SD) rate of 5.0 ± 1.4 atmol/min/rbc (red blood cell). Alko-keto reductase-catalyzed reduction of aldophosphamide (160 μM) to alcophabosphamide occurred at a much slower rate, viz. 0.3 ± 0.2 atmol/min/rbc. Thus, at a pharmacologically relevant concentration of aldophosphamide, viz. 1 μM, estimated aggregate erythrocyte ALDH-1-catalyzed aldophosphamide oxidation, viz. 2.0 μmol/min, was only about 3% of estimated aggregate hepatic enzyme-catalyzed aldophosphamide oxidation, viz. 72 μmol/min; however, this rate is greater than the estimated flow-limited rate of aldophosphamide delivery to the liver by the blood, viz. 1.5 μmol/min. These observations/considerations suggest an important in vivo role for erythrocyte ALDH-1 in systemic aldophosphamide detoxification. Erythrocyte ALDH-1 effects oxidation of other aldehydes to their corresponding acids, e.g. retinaldehyde to retinoic acid, may also be of pharmacological and/or physiological significance since a wide variety of aldehydes are known to be substrates for ALDH-1.

The therapeutically important circulating metabolite of the anticancer drug cyclophosphamide is 4-hydroxycyclophosphamide/aldophosphamide (reviewed in ref. 1). NAD(P)-linked aldehyde dehydrogenases catalyze the oxidation of aldophosphamide to carboxyphosphamide, thereby irreversibly detoxifying it (reviewed in ref. 1). Most efficient at doing so appears to be ALDH-1.1 Highest levels of this enzyme are ordinarily found in the liver. Thus, systemic detoxification of cyclophosphamide is thought to be catalyzed largely by hepatic ALDH-1, and, indeed, it catalyzes the majority (> 80% when the aldophosphamide concentration is pharmacological) of human hepatic aldophosphamide oxidation (2). However, lower levels of ALDH-1 are present in numerous tissues including erythrocytes (reviewed in ref. 3) where it reportedly accounts for the vast majority of aldehyde dehydrogenase-catalyzed acetalddehyde oxidation (reviewed in ref. 4). Since erythrocytes are continuously exposed to plasma 4-hydroxycyclophosphamide/aldophosphamide, the expectation is that some of it, known to be un-ionized and thereby irreversibly detoxifying it, is carried to the marrow and other organs of recipients who had been depleted of vital hematopoietic progenitor cells and their progeny as a consequence of having been treated with high-dose chemotherapy and/or radiotherapy in the interim (11).

Unknown is to what extent erythrocyte ALDH-1 contributes to the overall systemic oxidation of circulating aldophosphamide, and, if the contribution is ordinarily significant, as was expected given the fore-going and the large number of erythrocytes that are present in the human body, to what extent it varies. Thus, aldehyde dehydrogenase-catalyzed oxidation (detoxification) of aldophosphamide in human erythrocytes was quantified in the present investigation to address these questions. Also quantified was aldo-keto reductase-catalyzed reduction of aldophosphamide to alcophabosphamide, since 1) aldophosphamide per se is without cytotoxic activity (reviewed in ref. 1), 2) aldo-keto reductases, e.g. aldehyde and aldose reductases, are known to be present in erythrocytes (12), and 3) both, identified, e.g. aldehyde and aldose reductases, and unidentified aldo-keto reductases are known to catalyze the reduction of aldophosphamide to alcophabosphamide (reviewed in ref. 1).
The concentration of 4-hydroxycyclophosphamide/4-thiocyclophosphamide/aldophosphamide/thiohemiacetal (HTAH) in erythrocytes exceeds that in plasma by about 1.8-fold (5). The concentrations of carboxyifosfamide and ifosfamide mustard (metabolites of another oxazaphosphorine, ifosfamide, that correspond to carboxyphosphamide and phosphoramide mustard, respectively) in erythrocytes each exceed those in plasma by about 2.6-fold (6). Given that formation of glutathione conjugates of 4-hydroxycyclophosphamide/aldophosphamide is favored in erythrocytes because the erythrocyte glutathione concentration (0, 3- 3 mM, is vastly greater, about three orders of magnitude, than the plasma glutathione concentration, 3 - 4 µM (7, 8) and that these conjugates are highly ionized at physiological pH and thus unable to cross membranes, the relatively greater concentration of HTAH in erythrocytes is as expected. Given that the higher concentrations of HTAH in erythrocytes, the presence of ALDH-1 in erythrocytes but not in plasma, and the fact that both are highly ionized at physiological pH, the relatively greater concentration of carboxyifosfamide (carboxycylophosphamide) and ifosfamide mustard (phosphoramide mustard) is also as expected. Not reflected in this scheme is the distribution of 4-hydroxycyclophosphamide/aldophosphamide and phosphoramide mustard between plasma proteins (reversibly bound) and plasma water. Whether 4-hydroxycyclophosphamide/aldophosphamide binds to specific sites on plasma proteins is uncertain; approximately 3% of plasma phosphoramide mustard appears to be reversibly bound to plasma proteins (reviewed in ref. 1). Uncertain, too, is whether the multidrug resistance protein (MRP), a membrane glycoprotein (about 190 kDa) belonging to the ATP-binding cassette (ABC) protein family and known to be present in erythrocytes and to promote the export of certain glutathione conjugates (9), promotes the export of 4-thiocyclophosphamide and/or aldophosphamide hemiacetal from erythrocytes. However, existing evidence suggests that MRP-mediated export of these conjugates from erythrocytes is likely to be minimal (10).

Materials and Methods

Materials. 4-Hydroperoxycyclophosphamide was provided by Dr. Jörge Pohl, Asta Medica AG, Frankfurt, Germany. CM-Sephadex C-50, an isoelectric point marker kit (pI 4.5 - 6.5), and the Sigma Diagnostics Total Hemoglobin Determination Kit were purchased from Sigma Chemical Co. (St. Louis, MO). Isotonic buffered saline (Hematall) was purchased from Fisher Scientific (Fairlawn, NJ). Ampholine PAGplates (pH 4.5 - 6.5) were purchased from Pharmacia Biotechnology (Piscataway, NJ). Centriprep-100 concentrators were purchased from Amicon Division, W. R. Grace & Co. (Danvers, MA). All other materials were obtained from standard commercial sources or were prepared as described previously (2, 13).

Aldophosphamide was generated in aqueous solution by chemical reduction of 4-hydroperoxycyclophosphamide using methyl sulfide (99%+) as reducing agent as described previously (2, 14).

Purified human hepatic ALDH-1 was prepared as described previously (2). Polyclonal antibodies against purified human liver ALDH-1 were obtained by immunization of egg-laying hens (White Leghorn) essentially by the method of Gassmann et al. (15). Hens were housed at the University of Minnesota Poultry Teaching and Research Facility under regular light cycles (16-hr photoperiod) with food and water ad libitum. ALDH-1 (133 µg/ml in 25 mM triethanolamine buffer, pH 7.2, containing 1 mM dithiothreitol, 0.1 mM EDTA, and 25% glycerol) was emulsified in an equal volume of Freund's complete adjuvant. The emulsion was injected into the pectoral muscles at each of two sites on each side (total of 1.5 ml containing 100 µg ALDH-1). On day 16, a second injection of ALDH-1 emulsified in Freund's incomplete adjuvant was prepared and given as described above (total of 0.57 ml containing 40 µg ALDH-1). The eggs were collected daily, marked, and stored at 4°C until use.

Antibodies (IgY) were isolated from pools of egg yolks by polyethylene glycol precipitation and were partially purified by DEAE column chromatography as described by Gassmann et al. (15). As judged by immunoblot analyses of either native or denatured proteins and by ELISA, the antibody preparation was specific for ALDH-1 in that it did not recognize (detect) human class 2 (ALDH-2) or class 3 (ALDH-3) aldehyde dehydrogenases (up to 1,000 and 500 ng of either in the immunoblot assays and ELISA, respectively), whereas it did recognize (detect) as little as 50 (immunoblot analyses) or 1 ng (ELISA) of ALDH-1.

Human blood samples (EDTA-anticoagulant) were obtained through the American Red Cross (St. Paul, MN) from random blood donors (20 males and 15 females) ranging from 20 to 59 years of age. Mean ± SD ages of male and female donors and of the total population were 42 ± 10, 34 ± 9, and 39 ± 10 years, respectively. Blood samples were kept at 4°C for less than 48 hr before use.

Hemoglobin-depleted human erythrocyte lysate concentrates were prepared as follows. Erythrocytes were separated from plasma by centrifugation (5000g for 10 min at 4°C) after which the plasma and the buffy coat were removed by aspiration. The remaining pellet was then resuspended in Hematall and the suspension was centrifuged as before. The supernatant was again discarded and the pellet (erythrocytes) was resuspended in Hematall to give a 40% hematocrit.
Erythrocytes were counted with the aid of a hemacytometer. Aliquots of the erythrocyte suspension were saved for determination of hemoglobin content; the remaining erythrocytes were lysed by addition of 4 volumes of cold, double-deionized water. The lysate was centrifuged at 20,000g for 30 min at 4°C, supernatant was collected, the pellet was washed once with 25% Hematall, the wash was pooled with the original supernatant, and the combined pool was equilibrated for 30 min at 4°C with one volume of an 80% suspension of CM Sephadex C-50 in a 20 mM 2-[N-morpholino]ethanesulfonic acid buffer, pH 6.0, containing 1 mM dithiothreitol and 0.1 mM EDTA (Buffer A) to remove hemoglobin (16). The equilibrated mixture was vacuum-filtered through Whatman #1 paper and the retentate was washed with an equal volume of Buffer A. The wash was pooled with the filtrate and the volume was reduced using an Amicon ultrafiltration stirred-cell apparatus fitted with a YM-30 membrane and pressurized under nitrogen. The recovery of aldehyde dehydrogenase and aldo-keto reductase activities was consistently > 98%. Aliquots of the resulting hemoglobin-depleted erythrocyte lysate concentrate were used for enzyme assays or were further concentrated with the aid of an Amicon Centricon-100 concentrator for use in isoelectric focusing.

**Protein and Hemoglobin Determinations.** Protein content was quantified by the Coomassie brilliant blue dye binding assay (17) with the aid of the commercially available Bio-Rad Protein Assay Reagent; bovine serum albumin was used as the standard. The hemoglobin content of washed erythrocytes was determined in duplicate for all samples by the cyanmethemoglobin protocol (18) using the commercially available Sigma Diagnostics Total Hemoglobin Determination Kit with methemoglobin as the standard.

**Enzyme assays.** Aldehyde dehydrogenase activity was quantified spectrophotometrically, essentially as described previously (2, 14). The reaction mixture (1 ml, pH 8.2) contained 4 mM NAD, 32 mM tetrasodium pyrophosphate, 0.1 mM pyrazole, 5 mM glutathione, 1 mM EDTA, the substrate of interest, and a hemoglobin-depleted erythrocyte lysate concentrate. The reaction was initiated by addition of aldehyde and was followed at 37°C by monitoring the appearance of NADH at 340 nm with a Beckman DU-70 recording spectrophotometer. Aldo-keto reductase activity was quantified spectrophotometrically, essentially as described previously (19). The reaction mixture (1 ml, pH 7.0) contained 160 μM NADPH, 100 mM sodium phosphate, 160 μM aldophosphamide, and a hemoglobin-depleted erythrocyte lysate concentrate. The reaction was initiated by addition of aldehyde and was followed at 37°C by monitoring the disappearance of NADPH at 340 nm with a Beckman DU-70 recording spectrophotometer.

Duplicate determinations of each enzyme activity were made for all samples. Preliminary experiments established that erythrocyte aldehyde dehydrogenase and aldo-keto reductase activities did not decline during the up to 48-hr period when blood was held at 4°C.

**Isoelectric Focusing, Enzyme Activity Staining, and Immunoblot Analysis.** Isoelectric focusing and staining for protein and aldehyde dehydrogenase activity was carried out as previously described (2) except that narrow range Ampholine plates (pH 4.5 - 6.5) were used, the cathode and anode buffers were 0.1 M β-alanine and 0.1 M glutamic acid/0.5 M phosphoric acid, respectively, and a narrow range standard protein pI marker kit was used to assign the pI values. Aldehyde dehydrogenase (4 mM) used as substrate to visualize aldehyde dehydrogenase activity on the focused gels. Immunoblot analysis was carried out essentially as described previously (13), except that the antibody and the blocking agent (5% instant nonfat milk) were in 0.01 M potassium phosphate buffer, pH 7.2, containing 0.1 M NaCl (Buffer B), and all washes were with Buffer B.

**Statistics.** The Student’s t-test was used to determine whether there were significant differences between groups.

**Results and Discussion**

Aldehyde dehydrogenase-catalyzed oxidation of acetaldehyde (160 μM) occurred at a mean rate of 220 nmol/min/g Hb (6.8 atmol/min/rbc), fig. 2. This value is in good agreement with those reported previously (20–22). Also in agreement with the reports of others (reviewed in refs. 3 and 4), catalytic activity distributed over an approximately 3-fold range (fig. 2) and was independent of the donors sex (p > 0.05, data not presented).

Aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide (160 μM) occurred at mean rates of 162 nmol/min/g Hb and 5.0 atmol/min/rbc (fig. 2). Again, catalytic activity distributed over an approximately 3-fold range. The rate of aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide obtained for individual erythrocyte samples was consistently about 70% that of aldehyde dehydrogenase-catalyzed oxidation of acetaldehyde, a value that is significantly different (p < 0.05) from that, viz. 106%, obtained with purified hepatic ALDH-1 under identical assay conditions.2

Subjecting the 35 hemoglobin-depleted erythrocyte lysate concentrates to isoelectric focusing invariably revealed the presence of one major and two minor bands semiquantifying enzyme-catalyzed oxidation of acetaldehyde; representative findings are shown in fig. 3.

A hemoglobin-depleted erythrocyte lysate concentrate was subjected to isoelectric focusing and immunoblot analysis. The amount of concentrate loaded onto the gel was sufficient to generate 5–10 nmol NADH/min as determined by a spectrophotometric aldehyde dehydrogenase assay when acetaldehyde (4 mM) was used as the substrate. Lane a, pl standards visualized, after isoelectric focusing, with the aid of Coomassie Brilliant Blue R-250. Lane b, aldehyde dehydrogenase(s) in the erythrocyte lysate concentrate visualized, after isoelectric focusing, with the aid of a nitroblue tetrazolium-based enzyme activity stain. Lane c, ALDH-1 in the erythrocyte lysate concentrate visualized, after isoelectric focusing and electrophoresis to Immobilon-PVDF transfer membranes, with the aid of polyclonal antibodies raised to purified liver ALDH-1. Additional details are presented in Materials and Methods.

The pl value of the major band was 5.2 (fig. 3, lane b), a value that is identical to that previously reported for human liver ALDH-1 (2, 23). The pl values of the minor bands were 5.15 and 5.08. These values do not correspond to those of any known aldehyde dehydrogenases. Immunoblot analysis showed that all three bands were recognized by an antibody preparation raised to purified human liver ALDH-1 (fig. 3, lane c). It is likely that the two minor bands are slightly degraded products of ALDH-1 that differentially retain their ability to catalyze acetaldehyde oxidation as compared with their ability to catalyze aldophosphamide oxidation, thereby providing an explanation for the discrepancy noted in the previous paragraph.

These observations are consistent with the notion that erythrocyte ALDH-1-catalyzed oxidation of aldophosphamide contributes significantly to the overall systemic detoxification of circulating aldophosphamide. Pharmacologically relevant plasma concentrations of 4-hydroxycyclophosphamide/aldophosphamide are in the range of 0.1–10 μM (reviewed in ref. 1). The $K_m$ value for ALDH-1-catalyzed oxidation of aldophosphamide is 52 μM (2). Thus, at a saturating NAD concentration and an aldophosphamide concentration of 1 μM, erythrocyte ALDH-1 would be expected to catalyze the oxidation of aldophosphamide at a rate that is 2.5% of that at which it would catalyze the reaction if the aldophosphamide concentration were 160 μM, i.e. at a rate of 0.125 atmol/min/rbc. There are approximately $3.3 \times 10^{13}$ erythrocytes in an average 70-kg male. Collectively, they would be expected to oxidize 4.1 μmol aldophosphamide/min at saturating concentrations of NAD. The NAD level in erythrocytes is about 50 μM (24). The $K_m$ value for aldehyde-linked ALDH-1 catalyzed reduction of NAD is also about 50 μM (25). Thus, erythrocyte ALDH-1-catalyzed oxidation of circulating aldophosphamide would be at about 2 (range: 1.1–3.5) μmol/min when the aldophosphamide concentration is 1 μM. Not taken into account in these calculations is that 4-hydroxycyclophosphamide/4-thiocyclophosphamide/aldophosphamide/aldophosphamide thiohemiacetal is concentrated in erythrocytes (fig. 1). Systemic detoxification of aldophosphamide is generally viewed as being effected largely by hepatic enzymes, principally ALDH-1. Assuming a typical weight of 1500 g in an average 70-kg man, the liver would be expected to catalyze the oxidation of aldophosphamide at a rate of about 72 μmol/min when the aldophosphamide concentration is 1 μM, given that the $K_m$ defining this reaction is 52 μM, that catalysis occurs at a rate of 2.1 μmol/min/g liver when the aldophosphamide concentration is 160 μM and the NAD concentration is saturating (2), and that the hepatic NAD concentration is about 500 μM (26). However, hepatic oxidation of circulating aldophosphamide is probably much less rapid than this estimate because the rate at which it occurs depends, in large part, on the flow-limited rate of aldophosphamide delivery to the liver by the blood. Blood flow to the liver is typically about 1500 ml/min. Thus, at a concentration of 1 μM, delivery of aldophosphamide to the liver by the blood occurs at a rate of 1.5 μmol/min, a maximum clearance rate that is less than the aggregate erythrocyte mean clearance rate of 2.0 μmol/min, vide supra.

Catalysis of cyclophosphamide hydroxylation to yield 4-hydroxy-4-cyclophosphamide/aldophosphamide is largely, if not exclusively, by hepatic microsomal mixed-function oxidases (reviewed in ref. 1). Thus, it is likely that some of the aldophosphamide is oxidized to carboxyphosphamide before it ever leaves the liver and enters the general circulation. At first glance, this likelihood is seemingly inappropriately omitted from the foregoing deliberations. However, only the aldophosphamide that leaves the liver and enters the general circulation is ordinarily of any major importance since only it has any therapeutic or toxic potential towards any organ/tissue/cell other than the liver. In that context, then, the omission is justified.

Erythrocyte ALDH-1-effected oxidation of other aldehydes to their corresponding acids may also be of pharmacological and/or physiological significance since a wide variety of aldehydes are known to be substrates for ALDH-1 (reviewed in refs. 3, 27).

Also evident from these experiments is that care must be taken to remove all erythrocytes from tissues/organs that are to be evaluated with regard to the levels of ALDH-1 therein.

As judged by altered electrophoretic and/or kinetic properties, variants of ALDH-1 have been identified in human tissues, although
the incidence is apparently very rare (reviewed in ref. 3). Unknown is whether the variant forms of ALDH-1 catalyze the oxidation of aldophosphamide. As judged by isoelectric focusing and catalytic activity, there were no variants in our sample population of 35.

Erythrocyte ALDH-1 activity is apparently reduced or inhibited in a number of populations, e.g. in chronic alcoholics (reviewed in ref. 4), cigarette smokers (28, 29), snuff users (28), and users of birth control pills (30). Further, a number of frequently used drugs such as nitrate-ester antianginals, sulfonylurea hypoglycemics, certain cephalosporins, and disulfiram are known to inhibit ALDH-1, while diazepam and chlordiazepoxide seem to activate it (reviewed in refs. 4 and 31). Thus, there is the potential for clinically favorable/adverse drug interactions. Interindividual differences in the balance of urinary aldophosphamide metabolites have been reported; in some cases, there was a complete absence of carboxyphosphamide, suggesting a deficiency in relevant aldehyde dehydrogenase activity (reviewed in ref. 1).

Given: 1) the estimated minimum mean contribution of erythrocyte ALDH-1 to the systemic detoxification of circulating 4-hydroxycylophosphamide/aldophosphamide, 2) the 3-fold variation in constitutive levels of erythrocyte ALDH-1, 3) the potential for activating and inhibiting this enzyme, and 4) the inverse relationship between ALDH-1 levels and AUC values for 4-hydroxycylophosphamide/aldophosphamide, and, therefore, the therapeutic efficacy of cyclophosphamide (1), it follows that some of the variation in the therapeutic efficacy of cyclophosphamide experienced clinically may be the consequence of variation in the functional ALDH-1 content of erythrocytes. In that case, dosage adjustments based on the functional ALDH-1 content of erythrocytes may be warranted. These notions are, however, yet to be directly validated experimentally.

Scatter plots of individual values obtained for NADPH-linked aldo-keto reductase-catalyzed reduction of aldophosphamide (160 nM) are shown in fig. 4. Mean rates were 8.6 nmol/min/g Hb and 0.3 atmol/min/rbc, and were independent of the donors sex (p > 0.1; data not presented).

It is unlikely that NADPH-linked aldo-keto reductase-catalyzed reduction of aldophosphamide in erythrocytes contributes significantly to the systemic detoxification of aldophosphamide for several reasons: 1) in erythrocytes, reduction occurs at a rate approximately one order of magnitude slower than that of NAD-linked ALDH-1-catalyzed oxidation, e.g. the rate of reduction at 160 nM is about 5% of the rate of oxidation, as judged by in vitro analysis; 2) the $K_m$ values of 0.15 and 1.6 mM (19) for aldose and aldehyde reductase-catalyzed reduction of aldophosphamide, respectively, are higher than the $K_m$ value of 52 nM (2) for ALDH-1-catalyzed oxidation of aldophosphamide; thus oxidation would also predominate at pharmacological aldophosphamide concentrations; 3) reduction generates a metabolite, viz. alcopshamide, which retains cytotoxic potential, i.e. it can be reoxidized to aldophosphamide or directly converted to phosphoramid mustard (reviewed in ref. 1); oxidation is irreversible and the resulting metabolite, viz. carboxyphosphamide, lacks cytotoxic potential (reviewed in refs. 1 and 32).

Intact erythrocytes also catalyze the oxidation and reduction of retinaldehyde, viz. 0.20 and 0.02 atmol/min/rbc, respectively.3 It is likely that erythrocyte ALDH-1 and aldose reductase contribute to the oxidation and reduction of retinaldehyde, respectively, as retinaldehyde is a relatively good substrate for both of these enzymes. $K_m$ values are 0.3 μM (2) and < 1 μM (33), respectively. However, the physiological significance of these reactions is unclear, as the erythrocytes are not a target tissue for the retinoids, and retinol, not retinaldehyde, is the predominant circulating retinoid (34).

References


