An Additional Mechanism for the Cytotoxicity of 2-Chloroethylethyl Sulfide in Spleen Lymphocytes; Lysosomal Labilization

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Abstract: Exposure of spleen lymphocytes to 2-chloroethylethyl sulfide (CEES) leads to a reduction of the intracellular ATP level, followed by a decrease in cell viability. Addition of nicotinamide, an inhibitor of poly(ADP-ribose) polymerase (PADPRP), restores both ATP level and viability, indicating that an activation of PADPRP is responsible for the cytotoxicity of CEES. The involvement of a Ca^{2+}-mediated process in cytotoxicity is suggested. Verapamil, EGTA, trifluoperazine, and butacaine exhibit a partial protection (20 to 58%) against the cytotoxicity of CEES. Investigation of the causative role of proteolytic degradation in cell death indicate that pepstatin and leupeptin exert a substantial protective effect (60 to 70%), suggesting the involvement of lysosomal destabilization in CEES-induced cytotoxicity. Also, lysosomotropic agents markedly decrease the cytotoxicity. Lysosomal labilization may be a mechanism for the cytotoxicity of CEES.
Key words: 2-Chloroethylethyl sulfide (CEES), cytotoxicity, lysosome.

Recently, interest in vesicants, such as 2-chloroethylethyl sulfide (CEES) and 2,2′-dichlorodiethyl sulfide has increased. In addition to a vesicating action these compounds are mutagenic, carcinogenic, and cytotoxic. However, the biochemical basis for tissue injury resulting from exposure to vesicants is not completely understood. In blood lymphocytes (Meier *et al.*, 1987) sulfur mustards cause cross-linking of DNA strands, leading to activation of poly(ADP-ribose) polymerase (PADPRP), and eventually to depletion of NAD^{+} and ATP, which may be responsible for cell injury. An additional mechanism of ATP depletion was recently proposed since ATP loss in keratinocytes is not prevented by inhibitors of PADPRP (Margaret and Smith, 1993). An alternative hypothesis of Ca^{2+}-mediated cytotoxicity (Ray *et al.*, 1992) has been proposed in which alkylating agents readily react with glutathione (GSH), and a decrease in the intracelluar GSH level leading to lowered Ca^{2+}-ATPase activity, followed by an increase in intracelluar Ca^{2+} level.

Recent studies have indicated that a single subcutaneous injection of butyl 2-chloroethyl sulfide results in oxidative-type damage characterized by lipid peroxidation and depletion of the GSH content in brain and lung tissues of mice (Elsyaed *et al.*, 1989; Elsayed *et al.*, 1992). Membrane components have been mentioned as potential sites for alkylating agents, such as tris (2-chloroethyl)amine (Wildenaner *et al.*, 1980). In irradiated cells (Wills and Wilkinson, 1967) lysosomal membranes were susceptible to damage by radicals, and hydrolytic enzymes released from lysosomes were suggested to destroy cytoskeletal or nuclear components. In this respect, it was supposed that damage of lysosomal membrane could be induced by radiomimetic alkylating agents.

In this study, Lysosomal labilization is shown to be a cytotoxic mechanism of CEES in spleen lymphocytes, in addition to activation of PADPRP and a Ca^{2+}-mediated process.

Materials and Methods

Materials

2-Chloroethylethyl sulfide (CEES) was provided by Aldrich Chemical Co. (Milwakee, WI). All other reagents, including RPMI 1640 medium, were obtained from Sigma Chemical Co. (St. Louis, MD). Fetal calf serum was provided by Difco Laboratories (Detroit, MI).

Culture of spleen lymphocytes

Lymphocytes were prepared from ICR mice spleen (28 to 32 g) as described by Rosenberg and Lafrage-Fayssinet, (1983). Cultures were performed in 1 ml

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aliquots at a cell density of 1×10^6/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. The viability of cells was determined according to the trypan blue exclusion method (Meier and Johnson, 1992).

Exposure of lymphocytes to CEES

2-Chloroethylethyl sulfide (1 mM final conc.) dissolved in ethanol was added directly to the culture tubes containing lymphocytes (1×10^6/ml) at room temperature in a hood. The tubes were incubated for 1 h in a controlled environmental incubator (37℃ and 5% CO_2).

Protection of lymphocytes against CEES toxicity

Spleen lymphocytes in culture tubes were incubated with each candidate protective agent for 5 min before exposure to CEES (1 mM). After 1 h of exposure to CEES, cell viability was determined and expressed as a percentage of the control without CEES. The value was expressed as the mean± S.E. of three or more determinations.

Exposure of lysosomes to CEES

Aliquots (150 μl) of the lysosome-rich fraction (Smith *et al.*, 1976) were incubated with CEES dissolved in ethanol in 2 ml of 40 mM Tris buffer (pH 7.4) containing 0.2 M sucrose at 37℃. After 30 min of incubation the mixtures were chilled and centrifuged at 27,000 x g at 4℃. The supernatants were used for determination of arylsulfatase activity (Roy, 1953). The release of enzyme was expressed as a percentage of the value achievable after treatment with 0.2% Triton X-100. Each value was the average of three determinations.

Table 1. Effect of Ca^{2+}-channel blocker or Ca^{2+}-chelator on the CEES-induced cytotoxicity. Spleen lymphocytes (1×10^6/ml) in RPMI 1640 medium was exposed to 1 mM CEES for 1 h in the presence of verapamil, EGTA or trifluoperazine. The viability was determined as described in Marerials and Methods. Protection (%) was expressed as the value of [(viability in the presence of CEES and each candidate compound − viability in the presence of CEES only)/(viability in the absence of CEES − viability in the presence of CEES only)]×100

Compound	Concentration (μM)	Viability (% of control)	Protection (%)
CEES only		63.2± 3.2	
+Verapamil	0.5	65.2± 2.6	5.4
	2	73.4± 10.0	27.7
+EGTA	300	65.2± 2.3	5.4
	1000	70.6± 1.1	20.1
	3000	71.2± 2.6	21.7
+Trifluoperazine	0.1	71.9± 4.6	23.6
	0.5	74.1± 5.2	29.6

Table 2. Effect of phospholipase A_2 inhibitors on the CEES-induced cytotoxicity. Spleen lymphocytes (1×10^6/ml) in RPMI 1640 medium were exposed to 1 mM CEES for 1 h in the presence of either butacaine or bromophenacyl bromide. The viability was determined as described in Marerials and Methods

Compound	Concentration (μM)	Viability (% of control)	Protection (%)
CEES only		63.2± 3.2	
+Butacaine	10	81.2± 3.1	48.9
	30	84.6± 5.0	58.2
	60	77.8± 2.4	39.7
+Bromophenacyl bromide	10	32.9± 0.7	−82.3
	30	45.2± 2.7	−48.9
	100	46.5± 2.7	−45.4

Results and Discussion

When spleen lymphocytes were incubated with 1 mM CEES at 37℃, the level of intracelluar ATP and the viability were found to decrease gradually to approximately 24% and 63% of the control level after 1 h, respectively. In the presence of 1 mM nicotinamide, an inhibitor of PADPRP, the ATP level and the cell viability were 75% and 84% of the control, respectively. These results are consistent with previous studies using peripheral blood lymphocytes (Meier *et al.*, 1987), suggesting that activation of PADPRP results in a loss of ATP followed by a decrease of viable lymphocytes. Mol *et al.* (1989) showed that the protective role of nicotinamide was not demonstrated in keratinocytes with a low level of PADPRP activity. Thus, the vesicating action of CEES was not related to the activation of PADPRP. Therefore, it was attempted to protect lymphocytes against CEES cytotoxicity without interfering with PADPRP activity.

Since exposure to CEES increases the intracelluar Ca^{2+} level in intact cells (Ray *et al.*, 1992), various Ca^{2+}-related compounds were tested for an ability to protect against CEES cytotoxity. As shown in Table 1, verapamil (2 μM), a Ca^{2+}-channel blocker, and EGTA (3 mM) as a Ca^{2+}-chelator, expressed partial protection levels of 28% and 22%, respectively. Also, trifluoperazine (0.5 μM), a calmodulin antagonist, showed some protective ability (30%). These results suggest that a Ca^{2+}-mediated process might be responsible for some part of CEES cytotoxicity in spleen lymphocytes. Therefore, Ca^{2+}-dependent hydrolases, such as phospholipase A_2 (PLA_2) and Ca^{2+}-protease, may be involved in the process of cell death. As shown in Table 2, butacaine, an inhibitor of PLA_2, exhibited an appro-

Table 3. Effect of protease inhibitors on CEES-induced cytotoxicity. Spleen lymphocytes were exposed to 1 mM CEES for 1 h in the presence of the respective candidate compound. The viability was determined as described in Materials and Methods. Protection (%) was expressed as described in Table 1

Compound	Concentration (μM)	Viability (% of control)	Protection (%)
CEES only		63.2± 3.2	
+Leupeptin	10	72.6± 7.0	25.5
	30	84.2± 1.7	57.1
	60	88.3± 2.5	68.2
+Pepstatin	2	74.2± 1.7	29.9
	6	87.0± 2.2	64.7
	15	82.4± 4.7	52.2
+APMSF	2	66.2± 0.4	8.2
	6	58.2± 3.0	−13.3
	20	57.1± 4.7	−16.6
+DFP	20	61.8± 4.3	−3.8
	50	70.9± 2.6	20.9
	100	69.9± 3.6	18.2

ximately 22% increase in viability. However, no protective effect was observed with 30 μM bromophenacyl bromide. The inefficacy of bromophenacyl bromide might be due to its cytotoxicity in lymphocytes.

In a separate experiment (Table 3) where protease inhibtors were included, leupeptin, an inhibitor of cysteine protease, displayed a substantial protection (68%), whereas inhibitors of serine protease such as 4-aminophenylmethanesulfonyl fluoride (APMSF) and diisopropylfluorophosphate (DFP), exhibited only a small level of protection (8 to 20%), indicating that cysteine proteases may be more active than serine proteases in CEES cytotoxicity under the experimental conditions. Pepstatin, an inhibitor of aspartic protease, also demonstrated remarkable protecton (65%) against CEES cytotoxicity, although elastinal, an inhibitor of lysosomal elastase, had no effect. Since pepstatin had been reported to inhibit lysosomal aspartic protease, it was assumed that the lysosomal component might be a susceptible target for CEES cytotoxicity. To support this assumption, CEES cytotoxicity was examined in the presence of lysosomotropic agents (Gordon and Seglen, 1990). As shown in Table 4, chloroquine, a lysosomotropic hydrophobic amine at 10 μM, remarkably reduced cytotoxicity, although it seemed to show its own toxicity at concentrations higher than 100 μM. Methylamine, a lysosomotropic alkylamine at 5 mM, also expressed a remarkable protective action (approximately 58%). In comparison, the weak-base alkylamine seems to be as effective as are protease inhibitors. Thus, these data support the idea that CEES can cause cytotoxicity

Table 4. Effect of lysosomotropic agents on the CEES-induced cytotoxicity. Spleen lymphocytes were exposed to 1 mM CEES for 1 h in the presence of the respective candidate compound. The viability was determined as described in Materials and Methods

Compound	Concentration (μM)	Viability (% of control)	Protection (%)
CEES only		63.2± 3.2	
+Chloroquine	10	84.3± 8.7	57.3
	30	79.1± 5.4	43.2
	100	78.0± 2.3	40.2
+Methylamine	1000	66.1± 1.2	7.9
	2500	78.5± 2.3	41.6
	5000	84.6± 3.8	58.2

Table 5. Effect of protease inhibitors on the release of arylsulfatase from lysosomes. Lysosomal fraction from liver or lymphocytes was incubated with 2 mM CEES in either the presence or absence of each inhibitor for 30 min at 37℃. Seperately, the same experiment was carried out in the presence of 50 μM Ca^{2+}. The release of arylsulfatase was determined as described in Materials and Methods

Treatment	Concentration (μM)	Percent of total release	
		2 mM CEES	2 mM CEES+ 50 μM Ca^{2+}
Control	−	11.9(12.4)	26.4
Leupeptin	50	−	23.8
	100	−	20.0
	200	6.4(5.5)	18.6
Pepstatin	5	−	27.8
	15	11.7(13.3)	26.9

*The parenthesis indicates the value obtained with lysosome from lymphocytes.

by inducing destabilization of lysosomes in intact cells.

To investigate a direct effect of CEES on lysosomal membranes (Gross *et al.*, 1981), the lysosomal fraction from liver homogenate was exposed to 2 mM CEES in either the presence or absence of Ca^{2+}, and the release of arylsulfatase from lysosomes was determined (Table 5). Both Ca^{2+}-dependent and Ca^{2+}-independent release of arylsulfatase was partially inhibited by leupeptin, which showed a maximum inhibition at 200 μM. However, there was no inhibition by pepstatin (15 μM), suggesting that a cysteine protease may be directly involved in CEES-induced destabilization of lysosomes *in vitro*. The possibility that the *in vitro* lysosomal labilization test may be due to the presence of extralysosomal proteases is excluded, since α-macroglobulin (100 μg/ml), a general protease inhibitor (Starkey and Barett, 1977), failed to inhibit the release of arylsulfatase. Likewise, when lysosomes from lymphocytes were expo-

sed to 2 mM CEES, the release of arylsulfatase was also inhibited by leupeptin (200 μM). Although some aspect of lysosomal destabilization after exposure to CEES may be attributed to a proteolytic action of intralysosomal cysteine protease, the involvement of extralysosomal cysteine protease in intact cells is not excluded. Additionally, pepstatin-sensitive protease was not responsible for the direct effect of CEES. Although the mechanism for the protective action of pepstatin against CEES cytotoxicity was not further elucidated, pepstatin-sensitive protease in lysosomes may be indirectly involved in the cytotoxity of CEES. Therefore, CEES-induced lysosomal labilization in intact cells may be caused directly by a destabilization effect of CEES, and indiectly by an unknown biochemical process after CEES exposure.

The exposure of spleen lymphocytes to CEES may lead to activation of PADPRP activity, and a subsequent reduction in the ATP level and, eventually, an increase in the level of Ca^{2+}. This, in concert with a decrease in intracelluar pH, might enhance lysosomal labilization in intact cells.

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