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Inhibition of amino acid transport into lymphoid cells by the glutamine analog L-2-amino-4-oxo-5-chloropentanoate

(glutathione/1-chloro-2,4-dinitrobenzene/arginine)

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ABSTRACT **Transport of L-glutamine and of the chloroketone glutamine analog L-2-amino-4-oxo-5-chloropentanoate into lymphoid cells is mediated by the same system. Arginine and a number of other amino acids (e.g., glutamate, aspartate, and lysine) are transported to a much lesser extent by this system. However, after uptake of the chloroketone into the cells, the transport of glutamine, arginine, and other amino acids is markedly inhibited, due evidently to reaction of the chloroketone with intracellular components that are involved in amino acid transport. The chloroketone acts more effectively on growing than on resting cells. Treatment of lymphoid cells with the chloroketone or with 1-chloro-2,4-dinitrobenzene leads to rapid and complete depletion of intracellular glutathione without affecting cell viability. These reagents appear to be useful experimental tools for studies of glutathione function and metabolism.**

In the course of studies on the transport of glutamine into lymphoid cells, we examined the effects of L-2-amino-4-oxo-5-chloropentanoate. This chloroketone, which was first synthesized and used as a glutamine analog in studies on the reaction catalyzed by glutamine-dependent carbamyl-phosphate synthetase (1), was subsequently found to serve as an inhibitory glutamine analog for a number of glutamine amidotransferases (2–4). We now report evidence that the chloroketone is transported into lymphoid cells by the same system that transports glutamine and that the transport of the chloroketone into the cells leads to substantial inhibition of the transport of glutamine and also of other amino acids. The chloroketone competes for transport with glutamine but not with arginine, which is evidently transported by a different system. However, after transport of chloroketone into the cells, transport of glutamine, arginine, and a number of other amino acids is substantially inhibited. In this work we also found that treatment of lymphoid cells with the chloroketone leads to rapid and marked depletion of intracellular glutathione without significantly affecting cell viability. The findings suggest that the irreversible inhibition of glutamine transport by the chloroketone may be due to depletion of intracellular glutathione and also to interference with other cellular components that function in amino acid transport.

MATERIALS AND METHODS

Materials. The human lymphoid cell lines were obtained and maintained as described (5). L-2-Amino-4-oxo-5-chloropentanoate and L-2-amino-4-oxo-5-chloro[5-^{14}C]pentanoate were obtained as described (1, 2). 6-Diazo-5-oxo-L-norleucine was a gift from R. E. Handschumacher. 5-Diazo-4-oxo-L-norvaline was prepared as described (1, 2). ^{3}H- and ^{14}C-labeled L-amino acids were obtained from New England Nuclear.

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Treatment of Cells with the Chloroketone. Lymphoid cells (10^7/ml) suspended in phosphate-buffered saline (6) containing glucose (1 mg/ml) were incubated with shaking at 37°C in the presence and absence of chloroketone and amino acids as indicated. The cells were washed (10^7/ml) twice at 4°C with phosphate-buffered saline.

Amino Acid and Chloroketone Uptake Studies. The cells (10^7/ml) were suspended in phosphate-buffered saline containing glucose (1 mg/ml). Labeled amino acid (20 μl of a 0.2 mM solution containing 10 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) or chloroketone (2 μCi/μmol) was added to 0.2 ml of cell suspension. The suspension was incubated with shaking at 37°C. Aliquots (50 μl) were removed at 0, 1, and 3 min and layered on 0.1 ml of a 1:4 (vol/vol) mixture of mineral oil and dibutyl phthalate. After centrifugation in a plastic tube in a Beckman Microfuge for 0.3 min, the tip of the tube containing the cell pellet was cut off with a razor and the cells were suspended in 0.9 ml of 0.9% NaCl. After addition of 0.1 ml of 1 M NaOH and 10 ml of Bray's solution (7), radioactivity was determined by scintillation counting. Amino acid uptake is expressed as picomoles of amino acid taken up per mg of cellular protein (8) per min.

Determination of Glutathione. Cell suspensions (10^7/ml) were treated with trichloroacetic acid (final concentration, 5%); after centrifugation, the supernatant solution (1 ml) was extracted three times with 3–5 ml of ether to remove trichloroacetic acid, and total glutathione (oxidized and reduced) was then determined by the glutathione reductase method (9).

RESULTS

Effect of the Chloroketone on Uptake of L-Glutamine and Other Amino Acids by Lymphoid Cells. When lymphoid cells were incubated with 1 mM chloroketone for 30 min and then washed and resuspended in phosphate-buffered saline, there was a marked decrease (about 70%) in the rate of L-glutamine uptake (Table 1). Such treatment did not affect cell viability as determined by the trypan blue exclusion test. In similar studies with 1 mM 6-diazo-5-oxo-L-norleucine or 10 mM 5-diazo-4-oxo-L-norvaline, there was no inhibition. The marked inhibitory effect of the chloroketone was not observed when incubation of the cells with the chloroketone was carried out in the presence of L-glutamine or L-methionine. Other amino acids (e.g., L-leucine, L-valine, glycine, and L-phenylalanine) had some protective effect, but no protection against the effects of the chloroketone was found with L-arginine, L-glutamate, or L-lysine. In general, the relative effectiveness of various amino acids in protecting the cells against chloroketone-induced inhibition of L-glutamine uptake was parallel to the ability of these amino acids to inhibit L-glutamine uptake. Thus, when the uptake of 20 μM labeled L-glutamine was measured in the presence of 10 mM L-methionine, there was more than 70% inhibition of uptake of labeled L-glutamine. Some inhibition

Table 1. Decreased L-glutamine uptake after treatment of lymphoid cells with the chloroketone: Protection against chloroketone by certain amino acids

Amino acid added	Uptake of L-[^{14}C]glutamine pmol/mg protein per min	% of control
Control*	1230	[100]
None	406	33
L-Glutamine	1070	87
L-Methionine	1160	94
L-Leucine	834	68
L-Valine	752	61
Glycine	752	61
L-Phenylalanine	916	74
D-Methionine	678	55
L-Arginine	488	40
L-Glutamate	348	28
L-Aspartate	348	28
L-Lysine	414	34

The cells (RPMI 1788) were incubated with 1 mM chloroketone in the absence of (none) and in the presence of 10 mM amino acid for 30 min at 37°C; after the cells were washed, their ability to take up L-[^{14}C]glutamine was determined.
* Untreated cells; no amino acid was added.

of uptake was also observed in the presence of 10 mM L-valine, glycine, or L-phenylalanine, but addition of 10 mM L-arginine, L-glutamate, L-aspartate, or L-lysine did not inhibit the uptake of labeled L-glutamine.

Although L-arginine did not compete with L-glutamine for uptake (nor did L-arginine protect the cells against the effects of the chloroketone), treatment of the cells with the chloroketone markedly inhibited the uptake of L-arginine. Treatment with the chloroketone also inhibited the uptake of a number of other amino acids (Table 2). However, it is notable that the inhibition of L-arginine uptake observed in chloroketone-treated cells was prevented when treatment with the chloroketone was carried out in the presence of L-glutamine or L-methionine; when the chloroketone treatment was carried out in the presence of L-glutamate, L-arginine, or L-lysine, only slight protection was observed (Table 3).

The data show that (*i*) treatment of the cells with the chloroketone decreases the transport of amino acids that are transported by different systems (e.g., glutamine, arginine) and (*ii*) protection against the effects of the chloroketone on transport is afforded by L-glutamine and L-methionine, but much less so by other amino acids. These findings suggest that the inhibitory effect of the chloroketone is due to intracellular interactions. In the experiments given in Table 4, the uptake of [^{14}C]chloroketone by cells was markedly inhibited by L-glutamine and L-methionine, but much less so by L-glutamate and L-arginine. This result is similar to the findings given in Table 1 which show that L-glutamate and L-arginine did not protect significantly against chloroketone-inhibition of L-glutamine transport, whereas L-glutamine and L-methionine protected.

Table 2. Decreased uptake of several amino acids after treatment of lymphoid cells with the chloroketone

Amino acid	Amino acid uptake, pmol/mg protein per min: Control	Chloroketone-treated*
L-Glutamine	1220	501 (41)
L-Methionine	1810	1140 (63)
Glycine	204	69 (34)
L-Alanine	1570	645 (41)
L-Serine	1860	662 (36)
L-Leucine	1950	939 (48)
L-Phenylalanine	959	632 (66)
L-Glutamate	130	39 (30)
L-Lysine	272	126 (46)
L-Arginine	756	246 (33)

Control cells (RPMI 1788) and cells treated with 1 mM chloroketone and washed were examined for their ability to take up labeled amino acids.
* % of control value is given in parentheses.

Table 3. Decreased uptake of L-arginine by chloroketone-treated lymphoid cells: Protection against the chloroketone by certain amino acids

Amino acid added	Uptake of L-[^{3}H]arginine pmol/mg protein per min	% of control
Control*	483	[100]
None	159	33
L-Glutamine	484	100
L-Methionine	457	95
L-Glutamate	289	60
L-Arginine	217	45
L-Lysine	221	46

The cells (RPMI 1788) were treated with chloroketone in the absence of (none) and in the presence of 10 mM amino acid; after the cells were washed, their ability to take up L-[^{3}H]arginine was determined.
* Untreated cells; no amino acid was added.

Depletion of Intracellular Glutathione. Because there is interest in the role of glutathione in transport (10), we measured the glutathione content of cells treated with the chloroketone. We found that treatment of the cells with the chloroketone led to rapid and marked depletion of intracellular glutathione. Thus, after incubation of the cells with 1 mM chloroketone at 37°C for 1, 3, 5, and 10 min, we found 93%, 72%, 50%, and 0%, respectively, of the initial intracellular glutathione concentration. Furthermore, as shown in Table 5, this effect of the chloroketone was decreased substantially by the presence of the same amino acids that prevented inhibition of amino acid uptake (Tables 1 and 3) and the uptake of chloroketone (Table 4). In contrast, amino acids that did not exhibit these effects (e.g., L-arginine and L-glutamate) did not prevent the decline in glutathione level produced by treatment with the chloroketone.

Although the findings suggest an apparent relationship between chloroketone-induced inhibition of amino acid transport and depletion of intracellular glutathione, the known high chemical reactivity of the chloroketone makes it necessary to consider the possibility that it acts also at other intracellular sites. Furthermore, the uptake of chloroketone is substantially greater on a molar basis than the intracellular content of glutathione. We therefore sought to decrease glutathione levels by another

Table 4. Effect of amino acids on uptake of the chloroketone

Amino acid added	Uptake of chloroketone pmol/mg protein per min	% of control
None	4660	[100]
L-Glutamine	778	17
L-Methionine	376	8
L-Glutamate	3090	66
L-Arginine	2960	64

The cells (RPMI 1788) were incubated with 1 mM [^{14}C]chloroketone in the absence of (none) and in the presence of 10 mM amino acid, and the uptake of [^{14}C]chloroketone was determined.

Table 5. Depletion of intracellular glutathione by treatment with the chloroketone and protection against this effect by certain amino acids

Amino acid added	Glutathione	
	nmol/mg protein	% of control
Control*	12.0	[100]
None	0	0
L-Glutamine	5.5	46
L-Methionine	6.9	58
L-Leucine	4.7	39
L-Glutamate	0	0
L-Aspartate	0	0
L-Lysine	0	0
L-Arginine	0	0

The cells were treated with 1 mM chloroketone in the absence (none) and presence of 10 mM amino acid; after the cells were washed, the glutathione (oxidized and reduced) content was determined.
* Untreated cells.

means and found that brief incubation of the cells with 1-chloro-2,4-dinitrobenzene [a known substrate of glutathione S-transferase (11)] led to rapid and apparently complete disappearance of intracellular glutathione. 1-Chloro-2,4-dinitrobenzene was found to be a very highly active reagent for depletion of intracellular glutathione. Thus, a concentration of 5 μM depleted glutathione levels (from 16 μmol/mg of protein to virtually zero) within 5 min at 37°C. There was no loss of cell viability. The effects of treatment of the cells of two lymphoid cell lines with 1-chloro-2,4-dinitrobenzene or with the chloroketone on the intracellular glutathione content and on the rate of uptake of L-[^{14}C]glutamine are given in Table 6. The rate of transport of L-glutamine and the glutathione levels of the controls decreased with time, and both the chloroketone-treated cells and the cells treated with 1-chloro-2,4-dinitrobenzene exhibited rates of transport that were lower than those of the controls. However, neither type of treatment abolished transport, although, except for one experiment, no glutathione could be detected. The more marked decrease in the rate of transport exhibited by the chloroketone-treated cells indicates that the chloroketone probably reacts with cellular components other than glutathione which are involved in amino acid transport.

Table 6. Effects of treatment with the chloroketone and with 1-chloro-2,4-dinitrobenzene on glutathione levels and L-[^{14}C]glutamine uptake

Length of treatment, min	L-[^{14}C]Glutamine uptake, pmol/mg protein per min		
	Control	Chloroketone	1-Chloro-2,-4-dinitrobenzene
A 0	1130 (28.3)		
15	936 (18.4)	596 (0)	1060 (0.1)
30	698 (13.6)	189 (0)	493 (0)
60	548 (11.3)	101 (0)	425 (0)
B 0	970 (32.2)		
15	768 (17.1)	475 (0)	755 (0)
30	618 (15.2)	314 (0)	499 (0)
60	579 (12.7)	161 (0)	457 (0)

The cells (A, RPMI 6237; B, MOLT) were suspended in phosphate-buffered saline containing glucose (1 mg/ml) (control) or phosphate-buffered saline containing glucose and 0.5 mM chloroketone or 0.05 mM 1-chloro-2,4-dinitrobenzene. Aliquots (0.2 ml) were withdrawn for measurements of the rate of [^{14}C]glutamine uptake and intracellular glutathione concentration. Similar results were obtained with RPMI 1788 cells. Numbers in parentheses refer to glutathione level (nmol/mg of protein).

DISCUSSION

The present findings indicate that the chloroketone and glutamine compete for transport. Thus, glutamine protects against the inhibitory effects of chloroketone on amino acid transport and against its depleting effect on intracellular glutathione levels. In addition, glutamine markedly inhibits uptake of the chloroketone (Table 4). That cells treated with chloroketone exhibit reduced ability to transport many amino acids may be explained, as discussed above, in terms of a general effect of chloroketone on amino acid transport. Previous studies have shown inhibition of amino acid accumulation in rat kidney slices by diamide (12), a compound known to oxidize glutathione (13). In the present work, we found that both the rate of transport of L-glutamine and the intracellular content of glutathione declined in the controls (Table 6). Depletion of glutathione by treatment with the chloroketone or with 1-chloro-2,4-dinitrobenzene was also associated with decreased rates of glutamine transport. Depletion of glutathione by treatment of the cells with 1-chloro-2,4-dinitrobenzene may probably be ascribed to the activity of glutathione S-transferase, an activity present in substantial amounts in lymphoid cells (unpublished data). The decline in intracellular glutathione found after treatment with the chloroketone may be due to a similar mechanism and also to inhibition of γ-glutamylcysteine synthetase (14). The finding that treatment with the chloroketone, which also depletes glutathione, produces a greater decrease in glutamine uptake activity suggests that the chloroketone also interacts with other cellular components. One possibility is that the chloroketone reacts with an intracellular component associated with the cell membrane. For example, it has been found that sulfhydryl groups are involved in the polymerization of tubulin (15–17) and that the binding of colchicine to tubulin is decreased in tubulin whose sulfhydryl groups are blocked (16). Preliminary studies in this laboratory have shown that the binding of colchicine to extracts of chloroketone-treated lymphoid cells is reduced compared to untreated controls; on the other hand, treatment with 1-chloro-2,4-dinitrobenzene did not affect the binding of colchicine (unpublished data).

We have also found that the chloroketone exhibits a selective inhibitory effect on different lymphoid cells. Thus, glutamine uptake by phytohemagglutinin-induced lymphoblasts, as well as by various lymphoid cell lines, is inhibited by chloroketone treatment to a substantially greater extent than is that of resting peripheral lymphocytes. It seems possible that such differences in sensitivity to chloroketone inhibition reflect corresponding differences in glutamine uptake activity and therefore that the chloroketone (and perhaps similar compounds) exhibits a degree of selectivity that may be useful in certain experimental and therapeutic situations. Finally, it seems notable that treatment of lymphoid cells with chloroketone or with 1-chloro-2,4-dinitrobenzene leads to effective depletion of intracellular glutathione apparently without affecting cell viability. These reagents may therefore prove useful in experimental work on the function and metabolism of glutathione.

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