Characterization of Single Step Albizziin-resistant Chinese Hamster Ovary Cell Lines with Elevated Levels of Asparagine Synthetase Activity*

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The amino acid analog, albizzain, which acts as a competitive inhibitor of asparagine synthetase with respect to glutamine was used to isolate mutants of Chinese hamster ovary cells with alterations in levels of the target enzyme. These mutant lines have been characterized biochemically and genetically. Mutants selected in a single step are up to 40-fold more resistant to the drug than the parental line, express levels of asparagine synthetase activity 6–17-fold greater than that of wild type cells, and act co-dominantly in hybrids.

Several classes of mutations can be distinguished on the basis of cross-resistance to β-aspartyl hydroxamate, another amino acid analog. Studies on asparagine synthetase indicate that resistance to albizzain may be due to altered regulation of asparagine synthetase, structural mutations of the enzyme, and gene amplification.

As part of our interest in the study of the regulation of asparaginase synthetase activity we have isolated mutants of Chinese hamster ovary (CHO) cells resistant to two analogs, β-aspartyl hydroxamate (β-AHA), and albizzain (Alb), which show alterations in such regulation (1, 2). In the case of β-AHA, we (1) and others (3) have been able to obtain single-step lines which express constitutively elevated levels of asparagine synthetase. In order to obtain cell lines which greatly overproduce asparagine synthetase we utilized a selection scheme which has proven useful in the isolation of methotrexate (4) and N-phosphonacetyl-L-aspartate (5) resistant lines which have amplifications in the genes for the target enzymes, dihydrofolate reductase (6) and the multifunctional carbamyl phosphate synthetase-aspargyl-tRNA synthetase with respect to asparagine synthetase. We show as well that albizzain acts in vitro as a competitive inhibitor of asparagine synthetase with respect to glutamine and also as a competitive inhibitor of asparaginyl-tRNA synthetase with respect to asparagine.

EXPERIMENTAL PROCEDURES

Cell Culture—The parental cell lines used were five independent clones of wild type CHO cells and the GAT- cell line, a glycine, adenosine, and thymidine CHO auxotroph (14). All parental and single-step AlbR cell lines were routinely grown at 37 °C in α-complete medium (15) containing ribo- and deoxyribonucleosides supplemented with 7% heat-inactivated (65 °C for 30 min) fetal calf serum. Single-step albizzain-resistant lines (AlbR 1, 2, 3, 6, 10, 26, 27) were isolated from parental lines after ethylmethane sulfonate mutagenesis by growing cells in a complete medium lacking asparagine supplemented with 7% dialyzed fetal calf serum (medium 1) containing 2 mM albizzain as previously described (3). Multistep line (AlbR 42) was isolated in the same medium without prior EMS mutagenesis and subcultured into progressively increasing concentra-

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‡ The abbreviations used are: CHO, Chinese hamster ovary; β-AHA, β-aspartyl hydroxamate; Alb, albizzain.
tions of albizzin as previously described (2). The $D_{50}$ (concentration of drug required to reduce the relative plating efficiency to 10%) values were determined by plating 500 cells/10-cm dish in medium 1 containing various concentrations of the drug.

Hybrids were formed by the fusion of the Alb$^R$ lines which were isolated from the GAT$^+$ parental line and TsH1, a temperature-sensitive leucyl-tRNA synthetase CHO mutant (16) using polyethylene glycol 6000 (17), and growth at 38.5°C in medium 1 with reduced leucine and lacking thymidine and adenine.

**Determination of Enzyme Activities and Kinetic Constants** — Cell extracts were prepared from approximately 2 x $10^7$ cells, which had been washed in phosphate-buffered saline and resuspended in 0.4 ml of a buffer containing 1 mM dithiothreitol, 6 mM ethylenediaminetetraacetic acid, 30% (v/v) glycerol, and 50 mM Tris-HCl, pH 7.5. Cells were broken by freeze-thawing three times and centrifuged at 2800 x g for 10 min. The supernatants were used to assay asparagine synthetase or glutamine synthetase activity.

The activity of asparagine synthetase was measured by the conversion of L-[14C]aspartic acid to L-[14C]asparagine essentially as described by Gantt et al. (3) in an assay mixture which contained 120 mM Tris-HCl, pH 7.4, 5.5 mM ATP, 5.5 mM MgCl$_2$, 22 mM L-glutamine, and 2 mM L-[14C]aspartic acid (0.60 Ci/mmol). The final reaction mixture of 0.067 ml contained 120-400 μg of cell extract protein.

Glutamine synthetase activity was determined in a γ-glutamyl transfer reaction essentially as described by Rowe et al. (18) using hydroxylamine as substrate. The final volume of 1 ml contained 50 mM imidazole-HCl, pH 7.2, 5 mM MgCl$_2$, 50 mM L-glutamine, 20 mM sodium arsenate, pH 7.2, 0.1 mM ADP, 100 mM hydroxylamine, and 1 mg of cell extract protein. The reaction was terminated (after up to 90 min at 37°C) by the addition of 1.5 ml of ferric chloride solution (0.37 M FeCl$_3$, 0.67 N HCl, 0.2 M trichloroacetic acid), precipitated protein was removed by centrifugation, and the absorbance was read at 550 nm. One unit of activity catalyzes the formation of 1 μmol of γ-glutamylhydroxamic acid (absorbance of 0.34).

Asparaginyl-tRNA synthetase activity was determined by measuring the esterification of [14C]asparagine to tRNA as previously described (19).

Kinetic constants for asparagine synthetase and asparaginyl-tRNA synthetase were determined from double-reciprocal plots of initial velocity measurements of enzyme activity. For determination of inhibition of asparagine synthetase by albizzin with respect to glutamine, the albizzin concentration was varied from 0 to 30 mM and the glutamine concentration from 0 to 2 mM. The kinetic constants for asparaginyl-tRNA synthetase were determined in assay mixtures containing 0 to 10 mM albizzin and 0 to 25 μM asparagine.

**RESULTS**

**Degree of Resistance to Alb and Cross-resistance to β-AHA**

In order to examine the degree of resistance of the Alb$^R$ lines dose-response curves were used to determine $D_{50}$ values. As shown in Fig. 1 the $D_{50}$ of the parental line was 0.75 mM albizzin. Although the first step lines had been selected in 2 mM albizzin, they were resistant to much higher concentrations of the drug with $D_{50}$ values up to 40-fold greater than that of the parental line (Fig. 1 and Table I).

Albizzin and β-AHA affect the same target enzyme, and it might be expected that different types of alterations in asparagine synthetase would be reflected in different degrees of cross-resistance of the Alb$^R$ isolates to β-AHA. We, therefore, examined the cross-resistance of Alb$^R$ lines to β-AHA as shown in Table I. As previously described (1) and included in Table I (AH2), first-step β-AHA$^R$ mutants were only 3-fold more resistant to β-AHA than the parental line. The data in Table I show that all of the Alb$^R$ mutants were cross-resistant to β-AHA, but three classes could be distinguished according to this criteria. Most of the mutants were substantially more resistant than the parental line to both albizzin (16- to 40-fold) and β-AHA (4- to 6-fold). One mutant (Alb$^R$ 6) was highly resistant to albizzin (29-fold) but not to β-AHA (2-fold). Other less resistant mutants (Alb$^R$ 26, Alb$^R$ 27) were similar to the β-AHA$^R$ line.

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**Fig. 1. Dose-response curve for parental (O) and Alb$^R$ 1 (O), Alb$^R$ 6 (Δ), Alb$^R$ 10 (□), and Alb$^R$ 42 (○).**

**Table I**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Albizzin</th>
<th>β-Aspartyl hydroxamate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$D_{50}$</td>
<td>Increase$^a$</td>
</tr>
<tr>
<td>GAT$^+$</td>
<td>0.73</td>
<td>1</td>
</tr>
<tr>
<td>Alb$^R$ 1</td>
<td>18</td>
<td>25</td>
</tr>
<tr>
<td>Alb$^R$ 2</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>Alb$^R$ 3</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>Alb$^R$ 10</td>
<td>&gt;30</td>
<td>&gt;41</td>
</tr>
<tr>
<td>Alb$^R$ 42</td>
<td>21</td>
<td>29</td>
</tr>
<tr>
<td>Alb$^R$ 6</td>
<td>21</td>
<td>29</td>
</tr>
<tr>
<td>Alb$^R$ 26</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Alb$^R$ 27</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>AH2$^c$</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

$^a$ Relative to parental line.

$^b$ ND, not determined.

$^c$ β-Aspartyl hydroxamate-resistant CHO line.

**Dominance-recessiveness Test of Hybrids** — Since β-AHA resistance could be either co-dominant (20% of the mutants) or recessive in hybrids (1) it was of interest to determine whether albizzin resistance behaved in the same way. Dose-response curves of Alb$^R$ X TsH1 hybrid lines indicated that all of the mutations were co-dominant (Fig. 2) although the degree of co-dominance was not always correlated with the resistance of the Alb$^R$ line. For example, hybrids of single-step mutants (e.g. Alb$^R$ 6 X TsH1) were 2-fold more resistant than hybrids of the parental lines (GAT$^+$ X TsH1). However, hybrids of a multistep line (Alb$^R$ 42 X TsH1) were 18-fold more resistant (data not shown) even though the $D_{50}$ of both Alb$^R$ 6 and Alb$^R$ 42 were the same.

**Overproduction of Asparagine Synthetase** — Since albizzin is a glutamine analog, there are many possible sites of action of the drug. We were particularly interested in obtaining asparagine synthetase overproducers preferentially. For this reason, culture medium which contained glutamine and lacked asparagine was used in the selection. However, when asparagine synthetase activity was measured in each of the albizzin-
Asparagine Synthetase Activity in Albizziin-resistant Cells

FIG. 2. Dose-response curves for hybrids between parental and Alb* lines (•••), Hybrids of parental lines GAT- × TsH1 (C), Alb* 1 × TsH1 (O), Alb* 6 × TsH1 (▲), and Alb* 10 × TsH1 (■).

TABLE II
Increase in asparagine synthetase activity in albizziin-resistant cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Asparagine synthetase specific activity</th>
<th>Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental (GAT-)</td>
<td>106</td>
<td>1</td>
</tr>
<tr>
<td>Alb* 1</td>
<td>1,789</td>
<td>17</td>
</tr>
<tr>
<td>Alb* 2</td>
<td>1,144</td>
<td>11</td>
</tr>
<tr>
<td>Alb* 3</td>
<td>986</td>
<td>9</td>
</tr>
<tr>
<td>Alb* 6</td>
<td>1,230</td>
<td>12</td>
</tr>
<tr>
<td>Alb* 10</td>
<td>1,220</td>
<td>12</td>
</tr>
<tr>
<td>Alb* 27</td>
<td>807</td>
<td>8</td>
</tr>
<tr>
<td>Alb* 42</td>
<td>27,560</td>
<td>260</td>
</tr>
</tbody>
</table>

Table II shows that resistant lines two major classes could be distinguished. In one, no increase in activity was observed. In the other, asparagine synthetase activity was found to be elevated by more than 5-fold, and most of these lines had approximately 10-17-fold increases in enzyme activity (Table II).

Determination of Kinetic Constants of Asparagine Synthetase—To examine whether any of the mutants contained an altered form of asparagine synthetase, apparent KM and KI values were determined for the enzyme from cell extracts of the parent and representative mutants of the three β-AHA cross-resistance groups. Albizziin was found to be a competitive inhibitor of asparagine synthetase with respect to glutamine. Albeit in parental and mutant cell extracts (Fig. 3). Parental cells exhibited an apparent KM for glutamine of 6 × 10^-4 M and KI for albizziin of 9 × 10^-4 M. Several of the other mutants showed some increase in KI for albizziin, but these changes were less than 2-fold (Table III). However, the enzyme from one mutant (Alb* 6) exhibited an increase in apparent KM for glutamine and KI for albizziin of 3- and 5-fold, respectively (Fig. 3, Table III). Therefore, at least one mutant appears to have a structural change in asparagine synthetase.

Regulation of Asparagine Synthetase—In wild type CHO cells the basal level of activity of asparagine synthetase has been shown to increase 2-3-fold when asparagine is removed from the medium. These studies have also shown that asparagine synthetase levels are regulated by the extent of tRNA aminoacylation. It was of interest to determine whether the high levels of asparagine synthetase observed in the Alb* mutants were regulated in a manner similar to that of wild type cells. To examine whether the amount of asparagine in the medium affected the activities of asparagine synthetase in the mutants, cells were grown in the presence or absence of 33 μM asparagine. All of the mutants differed from the wild type in that they exhibited constitutively elevated levels of asparagine synthetase activity independent of the presence of asparagine (Table IV).

To investigate further the effects of albizziin on asparagine synthetase activity, we examined the possibility that in addition to acting as a competitive inhibitor of asparagine synthetase, glutamine, albizziin might also mimic the product asparagine and repress asparagine synthetase activ-
Asparagine Synthetase Activity in Albizziin-resistant Cells

**DISCUSSION**

We have previously reported that the amino acid analogs, \( \beta \)-AHA and Alb, can be used to obtain multistep resistant lines which overproduce asparagine synthetase (1, 2). The analogs appear to differ in their mechanisms of action since multistep \( \beta \)-AHA lines have a maximum of 20-fold enhancement of activity and tend to have structural changes in the enzyme (1), whereas Alb lines expressed up to 300-fold elevations in enzyme levels (2) and are due to gene amplification (8).

Since the selection procedure for obtaining multistep lines involved culturing the cells in continuously increasing concentrations of the drug, it is difficult to determine the primary nature of the lesions. We were interested, therefore, in studying the types of Alb mutants obtained in single-step isolations, where the analog would only be present during the initial course of the selection.

The work described in this paper has shown that single-step albizziin-resistant mutants of CHO cells can be divided into the following 4 classes. 1) There was at least one mutant with a structural gene alteration (Alb\(^{6}\)) in which asparagine synthetase had a higher apparent \( K_M \) for glutamine and \( K_I \) for albizziin. The increase in apparent \( K_M \) for glutamine may not be disadvantageous to the cell since the medium contains an excess of glutamine over other amino acids. 2) Among the mutants which overproduced asparagine synthetase, one (Alb\(^{10}\)) had elevated levels of the enzyme in the presence of asparagine which could be repressed by the addition of albizziin. 3) In other mutants (Alb\(^{1}\), Alb\(^{6}\)) all regulation of asparagine synthetase had been lost and the enzyme was constitutively overproduced. The consistent loss of regulation of enzyme activity is an interesting first step in albizziin resistance and may have some significance in respect to gene amplification. For example, high levels of cadmium resistance have been shown to be due to amplification of the genes for the metallothioneins (21). In wild type CHO cells the levels of metallothioneins are regulated by heavy metals and glucocorticoids (22). However, glucocorticoid regulation is lost in cells selected for resistance to cadmium. The loss of the regulation of enzyme activity in the single step albizziin mutants suggests that this may play some role in development of resistance to albizziin.

The relationship between the first three classes and those discerned by the cross-resistance patterns to \( \beta \)-AHA is not yet clear. Although most of the albizziin-resistant mutants were highly resistant to both albizziin and \( \beta \)-AHA suggesting a common site of action of the drugs and/or increase in amount of normal enzyme, some mutants did not follow this pattern. Alb\(^{6}\) was highly resistant to albizziin but only slightly cross-resistant to \( \beta \)-AHA. The alteration in enzyme affinity for glutamine and albizziin in Alb\(^{6}\) may not have caused a concomitant change in affinity for \( \beta \)-AHA. 4) Half of the albizziin-resistant lines did not have elevated levels of asparagine synthetase or glutamine synthetase (data not shown) and had to be affected in another function. We, therefore, examined other possible targets of albizziin which might lead to resistance. Albizziin was transported into CHO portional and mutant lines with similar kinetics. The drug apparently enters on the same transport system as asparagine and glutamine since transport of albizziin was inhibited by the addition of glutamine and transport of asparagine was inhibited by albizziin.

Experiments in vitro indicated that albizziin not only inhibited asparagine synthetase activity with respect to asparagine but in addition was itself acylated to tRNA. This supports the results in vivo which suggest that the analog mimics the effects of the amino acid asparagine. Wild type cells expressed elevated levels of asparagine synthetase in medium lacking asparagine but basal levels when albizziin was added. Albizziin may act in a manner similar to asparagine by acylation to tRNA and repression of asparagine synthetase activity. Nevertheless, none of the mutants appeared to be altered in asparaginyl-tRNA synthetase activity. There are several other enzymes (discussed below) whose activities have been shown to be affected by albizziin, and these may be the sites of lesions in this fourth class of mutants.

It is probable that some of the mutant classes of Alb\(^{6}\) cells have amplified copies of the asparagine synthetase gene. This is being examined presently using our cDNA probe for asparagine synthetase.

As indicated in the introduction, whereas the two drugs \( \beta \)-AHA and albizziin affect the same target enzyme, they have very different effects when used in continuous selection. It

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was initially expected that both analogs would select mutants in the population with similar types of lesions. We have found that this is clearly not the case.

The albizzin-resistant lines differed from \( \beta \)-AHA' lines in that the mutation was always co-dominant in hybrids; the Alb\(^a\) lines were more resistant to the drug and had higher levels of asparagine synthetase activity. In multistep \( \beta \)-AHA' lines, isolated after several months of growth in gradually increasing concentrations of the drug, the resistance was due to structural gene mutations (2). Multistep albizzin-resistant lines were isolated more quickly and expressed much higher elevations in asparagine synthetase activity due to gene amplification (8). The major difference may be due to the fact that albizzin is a glutamine analog which inhibits many glutamine-utilizing enzymes (23, 24) whereas \( \beta \)-AHA is an aspartic acid analog and probably has fewer target enzymes. We used a selection system containing \( \mu \) M glutamine and probably has fewer target enzymes. We used a selection system containing 2 mM glutamine and lacking asparagine to enrich for those cells which overproduced asparagine synthetase. However, when the glutamine concentration was lowered to 0.2 mM to reduce the effective albizzin concentration for selection of the multistep lines, we found increased expression of other enzyme activities in concert with that of asparagine synthetase. For example Alb\(^a\) 42 cells have elevations in the activity of glutamine synthetase\(^5\) an enzyme which is also inhibited by albizzin. Other glutamine-utilizing enzymes may be affected as well. It has been shown by Cooper and Meister that albizzin can also act as a substrate of glutamine transaminase (25), and this enzyme might be affected in some of the albizzin-resistant cell lines. In addition, glutamine is utilized in two steps of purine biosynthesis, those catalyzed by phosphoribosylpyrophosphate amidotransferase and formylglycinamide ribonucleotide formyltransferase. We have observed overproduction of phosphoribosylpyrophosphate amidotransferase in one highly resistant cell line\(^6\) and expect that some lines might also overproduce formylglycinamide ribonucleotide amidotransferase since albizzin has been shown to inhibit the latter irreversibly (26). It is interesting that two of the most well characterized gene amplification systems, those involving the dihydrofolate reductase and carbamyl phosphate synthetase, aspartate transcarbamylase, dihydro-orotase genes, overproduce enzymes involved in nucleotide biosynthesis (4, 5). Whether this proves to be the reason that \( \beta \)-AHA and Alb produce different effects remains to be elucidated. Although the initial effects of both drugs would be on protein synthesis, albizzin may exert a major effect on DNA synthesis since most of the Alb\(^a\) lines have chromosomal abnormalities (2) which were not observed in \( \beta \)-AHA' cells lines.\(^7\)

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\(^6\) C. Duff and I. Andrulis, unpublished observations.

The first-step isolates described here provide the necessary material to examine the different types of molecular changes which lead to Alb drug resistance and to gene amplification. As indicated previously, using mRNA from one of the amplified lines, we have recently cloned a cDNA for asparagine synthetase (8). We are in the process of using this to study the copy number of the gene and messenger RNA levels in the Alb\(^a\) and \( \beta \)-AHA' mutants.

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REFERENCES