The form of folate affects the mechanisms of methotrexate resistance in *Enterococcus faecium*

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Several mechanisms have been described to explain the resistance of cells to methotrexate (MTX); however, the basis for the heterogeneity of mechanisms has been obscure. It was hypothesized that the type of MTX resistance in a single species can be influenced by the form of extracellular folate supplied during the development of resistance. Two strains of MTX-resistant *Enterococcus faecium* were developed by transferring the bacteria to media containing increasing concentrations of MTX in the presence of constant concentrations of either 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid (5-HCO-H₄PteGlu) or pteroylglutamic acid (PteGlu). These resistant strains were designated *E. faecium*/MTX/5-HCO-H₄PteGlu and *E. faecium*/MTX/PteGlu, respectively. The mechanisms of MTX resistance included: (1) increased folic acid reductase (FAR) activity in both resistant strains but increased dihydrofolate reductase (DHFR) activity only in *E. faecium*/MTX/PteGlu; (2) decreased synthesis and intracellular retention of MTX containing two glutamyl residues; (3) decreased uptake of MTX accompanied by decreased uptake of folates; and (4) reduction of folate-binding capacity. Among these, the form of folate present in the media during the development of resistance affected DHFR and FAR activities and the transport of folates. These findings, together with data from other laboratories, suggest that it may be important to use a reduced form of folate, a more physiological form than oxidized PteGlu, in the media during the development of resistance for the study of the mechanisms of MTX resistance in cultured cells.

Keywords: methotrexate resistance, *Enterococcus faecium*, folate, dihydrofolate reductase, folate-binding protein

INTRODUCTION

Methotrexate (MTX), an antifolate, has been widely used for cancer treatment since its development and the identification of its biological activities (Franklin *et al.*, 1949). MTX is a classical type of inhibitor of dihydrofolate reductase (DHFR, 5,6,7,8-tetrahydrofolate-NADP⁺ oxidoreductase, EC 1.5.1.3; reactions 1 and 2, Fig. 1) (Blakley, 1984; Schweitzer *et al.*, 1990) and is

known to be metabolized to polyglutamyl forms after its incorporation into cells (Whitehead *et al.*, 1975). The development of resistance to MTX is one of the most serious problems in cancer chemotherapy. Many researchers have proposed possible mechanisms for the development of MTX resistance including one or a combination of the following mechanisms: (1) gene amplification of DHFR (Alt *et al.*, 1976; Haber *et al.*, 1981); (2) mutation to produce DHFR with decreased affinity to MTX (Haber *et al.*, 1981); (3) decreased activity of folylpolyglutamate synthetase (EC 6.3.2.17; reaction 3, Fig. 1) leading to reduced MTX polyglutamate synthesis (Jolivet *et al.*, 1982); (4) increased activity of folate conjugase (pteroyl-poly- γ -glutamyl carboxypeptidase, EC 3.4.19.9; reaction 4, Fig. 1) to

Abbreviations: DHFR, dihydrofolate reductase; FAR, folic acid reductase; FBP, folate-binding protein; MTX, methotrexate; PteGlu, pteroylglutamic acid; H₂PteGlu, dihydrofolate; H₄PteGlu, tetrahydrofolate; 5-HCO-H₄PteGlu, 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid.

Fig. 1. Effect of MTX on folate metabolism. 1, FAR; 2, DHFR; 3, folypolyglutamate synthetase; 4, folate conjugase; 5, thymidylate synthase. The broken lines indicate inhibition by MTX and MTXGlu_n.

reduce the glutamyl chain-length of MTX (Prescot & Affronti, 1968; Sur *et al.*, 1986); (5) increased synthesis of pteroylpolyglutamates for the maintenance of intracellular folate homeostasis (Roy *et al.*, 1995); (6) decreased transport of MTX into cells via either the low-affinity high-capacity reduced-folate carrier or by a reduction in expression of folate receptor (Antony, 1996); and (7) increased expression of the protein associated with multidrug resistance (Shen *et al.*, 1986).

Enterococcus (formerly *Streptococcus*) *faecium* ATCC 8043 is routinely used in the microbiological assay of folates (Tamura, 1990). Wild-type *E. faecium* does not survive in the presence of MTX, since dihydrofolate (H₂PteGlu), a product of thymidylate synthase (EC 2.1.1.45; reaction 5, Fig. 1), cannot be recycled to tetrahydrofolate (H₄PteGlu) due to the inhibition of DHFR. To determine folate concentrations in plasma containing MTX, Hutchison (1957) developed a strain of MTX-resistant *E. faecium*. Since then, the characterization of DHFR in MTX-resistant strains of *E. faecium* has been carried out by many investigators (Blakley, 1984). However, the mechanisms by which resistance to MTX develops in *E. faecium* have not yet been extensively studied and the effect of the form of folate on the development of MTX resistance has, to our knowledge, never been explored. We undertook this study to identify the mechanisms of MTX resistance in *E. faecium* and the effect of folate derivatives on its development.

METHODS

Strains and chemicals. Wild-type *E. faecium* (*E. faecium*/wild) was obtained from the American Type Culture Collection (Rockville, MD, USA) and folic acid assay medium from Difco. MTX, pteroylglutamic acid (PteGlu) and [6R,S]-5-

formyltetrahydropteroyl glutamic acid (5-HCO-H₄PteGlu) were purchased from Sigma. [3', 5', 7, 9-³H]5-HCO-H₄PteGlu (94 × 10¹⁰ Bq mmol⁻¹), [3', 5', 7, 9-³H]PteGlu (100 × 10¹⁰ Bq mmol⁻¹) and [3', 5', 7-³H]MTX (71 × 10¹⁰ Bq mmol⁻¹) were obtained from Moravsek. H₂PteGlu was prepared according to the method described by Futterman (1963). MTX containing a total of four glutamyl residues (MTXGlu₄) and pteroyl-triglutamate with the end glutamate labelled with ¹⁴C (PteGlu₃[¹⁴C]Glu) were synthesized by the solid-phase technique of Krumdieck & Baugh (1969). Chicken pancreas folate conjugase was partially purified as described by Leichter *et al.* (1977).

Two strains of MTX-resistant *E. faecium* were developed by transferring bacteria to successively greater concentrations of MTX. The strain in which MTX resistance was developed in the presence of PteGlu was designated *E. faecium*/MTX/PteGlu and that developed in the presence of 5-HCO-H₄PteGlu as *E. faecium*/MTX/5-HCO-H₄PteGlu. The initial concentrations of MTX for the development of MTX resistance were 200 pM and 2.0 pM for *E. faecium*/MTX/PteGlu and *E. faecium*/MTX/5-HCO-H₄PteGlu, respectively. The concentrations of 5-HCO-H₄PteGlu (5.4 nM) or PteGlu (11.3 nM) were kept constant during the development of MTX resistance. The response curves of these strains to either PteGlu or 5-HCO-H₄PteGlu with various concentrations of MTX were constructed using 96-well microplates and a plate reader (Model 2550, Bio-Rad) (Tamura, 1990).

Enzyme assays. For the determinations of activities of DHFR, folic acid reductase (FAR) and folate conjugase, *E. faecium*/MTX/PteGlu was incubated in approximately 1.0 litres of medium containing PteGlu (11.3 nM), and *E. faecium*/MTX/5-HCO-H₄PteGlu and *E. faecium*/wild in medium containing 5-HCO-H₄PteGlu (11.3 nM) without MTX for 16 h at 37 °C. The bacteria were independently harvested by centrifugation at 6000 g for 20 min at 4 °C, and washed twice with 50 ml 0.05 M potassium phosphate buffer (pH 6.5). They were suspended in 20 ml of the same buffer and the cell membranes disrupted using a French press followed by centrifugation at 12000 g for 20 min at 6 °C. The supernatant of each strain was kept at -70 °C and used as an enzyme source.

The activities of DHFR were measured in triplicate by the method described by Nixon & Blakley (1968) using H₂PteGlu as substrate. The reaction mixture contained 38 μM H₂PteGlu, 50 μM NADPH, 10 μM mercaptoethanol and 30 mM potassium phosphate buffer (pH 6.7), and the enzyme preparation in a total of 1.0 ml. The decrease in absorbance at 340 nm was monitored using a spectrophotometer with temperature control (37 °C) and a control module allowing absorbance to be recorded at 30 s intervals (Spectronic 601, Milton Roy). The molar absorption coefficient of 12300 M⁻¹ cm⁻¹ for the reaction at 340 nm was used to calculate the enzyme activity (Nixon & Blakley, 1968). The activities of FAR were determined in triplicate using [³H]PteGlu as substrate (Rothenberg, 1965). The standard incubation mixture consisted of 0.2 mM [³H]PteGlu, 1.0 mM NADPH, 100 mM potassium acetate buffer (pH 5.0), and 10–60 μl of enzyme in a total volume of 200 μl. The blank mixture contained no enzyme and the counts of the blank were subtracted for calculation of the enzyme activity. After incubation at 37 °C for 20 min, 40 μl 27 mM PteGlu, 10 μl glacial acetic acid and 40 μl 0.17 M zinc sulfate were added to the mixture, kept in ice water for 10 min and then centrifuged at 3000 g for 10 min at 4 °C. The supernatant (145 μl) was mixed with 2.0 ml counting fluid (Gold Ultima, Packard Instrument) and the radioactivity

measured using a scintillation counter (Model 1219 Rackbeta, LKB). Folate conjugase activity was measured in triplicate by the method of Krumdieck & Baugh (1970) using PteGlu₃[¹⁴C]Glu as substrate. The incubation was carried out up to a maximum of 6 h at 37 °C. Protein concentrations were measured using a Bio-Rad Protein Assay kit.

Metabolism of MTX. This was determined using cell extracts after MTX-resistant and wild-type *E. faecium* were individually incubated in the regular assay media containing 3.7×10^4 Bq (0.05 nmol) [³H]MTX (71×10^{10} Bq mmol⁻¹) and 11.0 nM folates for 18 h and washed using saline. The bacteria were disrupted in a French pressure cell, heated in boiling water for 10 min and centrifuged at 900 g for 5 min. The supernatants (1.0 ml each) were applied to a Sephadex G-25 column (1 × 120 cm) and eluted with 0.1 M potassium phosphate buffer (pH 6.0) (Shin *et al.*, 1974). Fractions (3.0 ml) were collected and the radioactivity measured. Extracellular fluids were also applied to a Sephadex G-25 column without prior treatment. MTX and MTXGlu₃ were used as markers and their elution was monitored by determining UV absorbance at 303 nm (Blakley, 1969).

Uptake of folates. The uptake of [³H]5-HCO-H₄PteGlu and [³H]PteGlu by the three strains of *E. faecium* was determined with and without MTX. *E. faecium* were harvested by centrifugation (1800 g for 15 min at 8 °C) after an 18 h incubation at 37 °C in 10.0 ml of media without MTX. The bacteria were washed twice with 0.14 M NaCl and suspended in 10.0 ml 0.1 M potassium phosphate buffer (pH 6.3) containing 56 mM glucose. The optical density of the bacterial suspension was measured at 600 nm using a microplate reader. This suspension (20 µl) was incubated in a total volume of 5.0 ml potassium phosphate buffer containing glucose as above at 37 °C for 2 min prior to the addition of 0.20×10^{10} Bq of either [³H]5-HCO-H₄PteGlu (94×10^{10} Bq mmol⁻¹) or [³H]PteGlu (100×10^{10} Bq mmol⁻¹). Aliquots (1.0 ml) were removed at various intervals (1–15 min) and filtered using a 0.22 µm microfilter (Millipore). After the bacteria on the microfilter had been washed three times with 1.0 ml ice-cold water, the filter was placed in counting fluid and the radioactivity measured. The uptake experiments were repeated three times and the means are presented. The term 'uptake' was defined as the amount of radioactivity found in the cells after washing and expressed as pmol per 10 min per OD₆₀₀ unit. The uptake of [³H]MTX was determined in a similar manner.

Folate-binding protein (FBP) assay. The determination of [³H]PteGlu-bound folate-binding protein was carried out using bacteria harvested by centrifuging (4000 g for 20 min at 8 °C) 1.1 litres of media containing each strain of *E. faecium* incubated for 18–24 h with 2.27 nM of either 5-HCO-H₄PteGlu or PteGlu without MTX. Cells were disrupted in a French pressure cell with 5.0 ml 0.1 M potassium phosphate buffer (pH 7.5). Each cell extract was mixed with an equal volume of buffer containing 4% (w/v) Triton X-100 and 0.1% (w/v) sodium azide. The mixtures were shipped from Birmingham to Indianapolis on wet ice. The mixtures were solubilized by shaking for 24 h at 4 °C. To dissociate endogenous folates bound to FBP, the mixtures were dialysed against 4.0 litres 50 mM sodium acetate (pH 4.5) for 3 d, followed by a second 3-d-dialysis against 3.0 litres 100 mM potassium phosphate buffer (pH 7.5) and a third dialysis against 4.0 litres 10 mM potassium phosphate buffer (pH 7.5). All dialysates contained 1% Triton X-100 and 0.05% sodium azide, and were changed every 12 h throughout dialysis. All dialysis procedures were carried out at 4 °C. The dialysed

samples were centrifuged at 13600 g for 15 min, and the concentrations of protein were measured in each supernatant (Smith *et al.*, 1985). Aliquots of each supernatant were incubated with 123 pmol [³H]PteGlu for 45 min at 37 °C and applied to a calibrated Sephacryl S-200 column (2.5 × 30.0 cm, Spectrum Medical Industries), which was eluted with 10 mM potassium phosphate buffer (pH 7.5) containing 1% Triton X-100 and 0.05% sodium azide at 4 °C (Verma & Antony, 1991). Fractions (2.75 ml) were collected and their radioactivity measured. The entire procedure was repeated and the data presented are the means of two experiments. For both Sephadex G-25 and Sephacryl S-200 columns, the void volume (V₀) was determined with blue dextran with a molecular mass of 2000 kDa.

RESULTS

MTX-resistant strains

As shown in Fig. 2, wild-type *E. faecium* were made resistant to a final concentration of 40 µM MTX by transferring bacteria daily in increasing concentrations of MTX with a constant concentration of 5-HCO-H₄PteGlu or PteGlu. It required 19 transfers to develop resistance to a final concentration of 40 µM MTX for *E. faecium*/MTX/5-HCO-H₄PteGlu, whereas only six transfers were required for *E. faecium*/MTX/PteGlu. The growth responses of these mutant strains and *E. faecium*/wild to 5-HCO-H₄PteGlu and PteGlu with varying concentrations of MTX (0–25 µM) are illustrated in Fig. 3(a–c). Although both MTX-resistant strains maintained their ability to respond to 5-HCO-H₄PteGlu, *E. faecium*/MTX/5-HCO-H₄PteGlu did not show any response to PteGlu in the presence of 25 µM MTX, and the ability of this strain to respond to PteGlu was substantially reduced even in the absence of MTX

Fig. 2. Development of MTX-resistant *E. faecium*. Wild-type *E. faecium* was initially grown in medium containing 200 pM MTX and 11.3 nM PteGlu (○) or in medium containing 2 pM MTX and 5.4 nM 5-HCO-H₄PteGlu (●). Bacteria which showed sufficient growth (OD₆₀₀ of 0.4) were transferred daily to media containing higher concentrations of MTX. The OD₆₀₀ of this transferred bacterial suspension was almost equal to that of the blank (approximately 0.1) since only 0.15 µl of the original bacterial suspension of the previous day was mixed with 300 µl medium. The vertical scale indicates the concentrations of MTX to which *E. faecium* developed resistance.

Fig. 3. Growth responses of MTX-resistant (a, b) and wild-type (c) *E. faecium* to 5-HCO-H₄PteGlu (—) and PteGlu (---) with 10 µM (●) or 25 µM (□) MTX or without MTX (○). (a) *E. faecium*/MTX/5-HCO-H₄PteGlu; (b) *E. faecium*/MTX/PteGlu; (c) *E. faecium*/wild.

(Fig. 3a). Furthermore, for this mutant strain, the concentration of PteGlu which gave half-maximum growth was 80 nM without MTX, which was significantly higher than that for *E. faecium*/wild (2.5 nM) and *E. faecium*/MTX/PteGlu (4.0 nM) (Table 1). *E. faecium*/MTX/PteGlu retained the ability to respond to PteGlu (Fig. 3b). As expected, MTX-sensitive *E. faecium*/wild did not respond to either 5-HCO-H₄PteGlu or PteGlu in the presence of MTX (Fig. 3c).

Enzyme activities

As shown in Table 1, the activities of DHFR and FAR and the uptakes of [³H]folates were affected by the form of folate in the media during the development of resistance. *E. faecium*/MTX/PteGlu had markedly increased activities of DHFR and FAR as compared to *E. faecium*/wild, whereas FAR activity in *E. faecium*/MTX/5-HCO-H₄PteGlu was three times higher than that in *E. faecium*/wild. Folate conjugase activity was not detected in any strain of *E. faecium* under the assay conditions employed in this study.

Metabolism of MTX

The Sephadex G-25 chromatograms of intracellular and extracellular derivatives of [³H]MTX in MTX-resistant and wild-type *E. faecium* are shown in Fig. 4 (a, b). The chromatographic data indicate that approximately 81 % of [³H]MTX incorporated into *E. faecium*/wild was

Table 1. Mechanisms of MTX resistance in *E. faecium*

	<i>E. faecium</i> / MTX/5-HCO-H ₄ PteGlu	<i>E. faecium</i> / MTX/PteGlu	<i>E. faecium</i> / wild
PteGlu concentration for half-maximum growth (nM)	80	4.0	2.5
DHFR activity [nmol H ₄ PteGlu formed (mg protein) ⁻¹]*	52 ± 13	589 ± 170	63 ± 24
FAR activity [pmol H ₄ PteGlu formed (mg protein) ⁻¹]*	9.0 ± 2.4	33.0 ± 9.9	3.0 ± 1.6
Folate conjugase activity (pmol mg ⁻¹ h ⁻¹)*	< 12	< 12	< 12
MTXGlu synthesis (% of intracellular counts found as MTXGlu)	5	8	81
MTXGlu retention (% of MTXGlu counts found intracellularly)	0	0	78
[³ H]5-HCO-H ₄ PteGlu uptake (% of uptake by <i>E. faecium</i> /wild)*†	25 ± 7	46 ± 8	100
[³ H]PteGlu uptake (% of uptake by <i>E. faecium</i> /wild)*†	10 ± 3	16 ± 7	100
[³ H]MTX uptake (% of uptake by <i>E. faecium</i> /wild)*†	27 ± 9	21 ± 6	100
[³ H]PteGlu-bound FBP [pmol (mg protein) ⁻¹]	0.63	0.27	6.7

* Data are means of two or three experiments.

† Uptake by *E. faecium*/wild (± SD) was 2.0 (± 0.3) pmol per 10 min per OD₆₀₀ unit for [³H]5-HCO-H₄PteGlu, 4.0 (± 0.9) pmol per 10 min per OD₆₀₀ unit for [³H]PteGlu and 0.69 (± 0.2) pmol per 10 min per OD₆₀₀ unit for [³H]MTX.

Fig. 4. Sephadex G-25 column chromatograms of intracellular (a) and extracellular (b) MTX derivatives from MTX-resistant (broken lines) and wild-type (solid lines) *E. faecium* incubated with [3 H]MTX for 18 h. The void volume, V_0 , of the column (1.0×120 cm) was 36 ml and the total volume, V_t , was 94 ml. Fractions of 3 ml were collected for measurement of radioactivity. —, *E. faecium*/MTX/5-HCO- H_4 PteGlu; ---, *E. faecium*/MTX/PteGlu; —, *E. faecium*/wild. Arrows indicate the elution positions of MTXGlu $_3$ and MTX.

metabolized to a polyglutamate form of MTX as a single peak, and approximately 78% of this form of MTX was retained in *E. faecium*/wild after 18 h of incubation (Table 1). In all media, the major radioactive peak in each of the three strains eluted at the position of MTX and a minor peak which corresponded to the intracellular peak found in *E. faecium*/wild (Fig. 4b). However, in both MTX-resistant strains radioactivity was detected only at the peak corresponding to MTX (Fig. 4a). Only 5–8% of MTX incorporated into the cells was metabolized to polyglutamyl MTX and all were recovered in the media (Table 1, Fig. 4b).

The peak of polyglutamyl MTX in *E. faecium*/wild was identified as MTXGlu (MTX containing two glutamyl residues in its moiety) by treating the compound with two types of folate conjugases. As shown in Fig. 5, the elution position of the peak did not essentially change after treatment with chicken pancreas folate conjugase,

Fig. 5. Sephadex G-25 column chromatograms of MTXGlu from *E. faecium*/wild after treatment with chicken pancreas folate conjugase (---) or human serum folate conjugase (—). The void volume, V_0 , of the column (1.0×120 cm) was 36 ml and the total volume, V_t , was 94 ml. Fractions of 3 ml were collected for measurement of radioactivity. Arrows indicate the elution positions of MTXGlu $_3$ and MTX.

which is known to hydrolyse polyglutamates to diglutamates. By contrast, there were two peaks of radioactivity at the elution positions of both MTXGlu and MTX after the original peak was treated with human serum folate conjugase, which is known to hydrolyse polyglutamates to monoglutamates (Tamura, 1990). These findings indicate that MTX was metabolized to MTXGlu in *E. faecium*. Furthermore, in all three strains of *E. faecium*, [3 H]4-amino-4-deoxy-10-methylpteroic acid, a degradation product of MTX having a K_{av} of over 4.0 as reported by Shin *et al.* (1974), was identified in the media (data not presented). However, there was no significant difference in the amount of radioactivity found in the peak of this degradation product among the three strains.

Uptake of folates

The uptakes of [3 H]5-HCO- H_4 PteGlu and [3 H]PteGlu were markedly reduced in both MTX-resistant strains as compared to *E. faecium*/wild. Furthermore, in *E. faecium*/MTX/5-HCO- H_4 PteGlu, the uptakes of [3 H]5-HCO- H_4 PteGlu and [3 H]PteGlu were approximately 54% and 63%, respectively, of those in *E. faecium*/MTX/PteGlu (Table 1). The uptakes of [3 H]MTX by both MTX-resistant strains were approximately 25% of that by *E. faecium*/wild. However, there was no difference in the uptakes of [3 H]MTX between the two mutant strains (Table 1).

Folate-binding protein

As shown in Fig. 6, an FBP from *E. faecium*/wild eluted at the void volume. This FBP showed a total [3 H]PteGlu binding capacity of $6.7 \text{ pmol (mg protein)}^{-1}$. This capacity was significantly greater than those in the MTX-resistant strains [$0.63 \text{ pmol (mg protein)}^{-1}$ for *E.*

Fig. 6. Sephacryl S-200 column (3.5 × 30 cm) chromatograms of FBPs obtained after solubilization of MTX-resistant (broken lines) and wild-type (solid lines) *E. faecium* with Triton X-100. Endogenous folate was removed from FBPs by extensive dialysis (see Methods). One hundred microlitres of this dialysed solution was incubated with an excess amount of [3 H]PteGlu and applied to the column. The FBP was eluted with 10 mM phosphate buffer (pH 7.5) containing 1% (w/v) Triton X-100. Fractions of 2.75 ml were collected for measurement of radioactivity. —, *E. faecium*/MTX/5-HCO-H₄PteGlu; ---, *E. faecium*/MTX/PteGlu; —, *E. faecium*/wild.

faecium/MTX/5-HCO-H₄PteGlu and 0.27 pmol (mg protein)⁻¹ for *E. faecium*/MTX/PteGlu]. These data indicate that the FBP(s) level or binding affinity in MTX-resistant *E. faecium* was substantially reduced when compared to *E. faecium*/wild.

DISCUSSION

Our data indicate that four major alterations occurred in MTX-resistant strains as compared to *E. faecium*/wild. These include: (1) increased DHFR and FAR activities in *E. faecium*/MTX/PteGlu; (2) decreased synthesis and intracellular retention of MTXGlu; (3) decreased uptake of MTX accompanied by decreased uptake of folates; and (4) reduced binding capacity of FBP. Among these four alterations, the activities of DHFR and FAR and the uptake of folates were markedly affected by the form of folate (either 5-HCO-H₄PteGlu or PteGlu) supplied in the media during the development of MTX resistance. Since the development of MTX resistance in *E. faecium*/MTX/5-HCO-H₄PteGlu required more transfers than did *E. faecium*/MTX/PteGlu, it is likely that the development of MTX resistance occurred as stepwise mutational events. This process more closely resembles naturally occurring resistance development, since 5-HCO-H₄PteGlu is a natural form of the vitamin, while PteGlu is a synthetic form which does not exist in nature.

To our knowledge, there have been only a limited number of studies on the mechanisms of MTX resistance in various mammalian cell lines using a reduced form of

folate as a source of folate in the medium during the development of resistance. Most studies were not designed to investigate the effect of the form of folate derivative on the mechanism of development of MTX resistance (Hakala *et al.*, 1961; Halpern *et al.*, 1975; van der Veer *et al.*, 1989; Jansen *et al.*, 1990; van der Laan *et al.*, 1991a, b; Houghton *et al.*, 1992; Miyachi *et al.*, 1992).

The phenomenon observed in this study may apply only to this organism; however, the use of a reduced form of folate rather than oxidized PteGlu may be important to closely mimic physiological conditions when studying the development and mechanisms of antifolate resistance in cultured mammalian cells. For example, Miyachi *et al.* (1992) evaluated the effect of 5-HCO-H₄PteGlu or PteGlu in the media on the development of MTX resistance using K526, a human leukaemia cell line. The MTX-resistant K526 developed in the presence of PteGlu showed only impaired MTX transport as a mechanism for MTX resistance, whereas the resistant K526 developed in the presence of 5-HCO-H₄PteGlu demonstrated only increased DHFR gene expression. We previously reported that the form of folate used during the development of trimetrexate-resistant *E. faecium* affected the activities of DHFR and FAR as well as the character of FAR (Tamura *et al.*, 1992). Thus, it is becoming clear that the form of folate likely plays an important role in the development of resistance to antifolates.

The decreased uptakes of [3 H]5-HCO-H₄PteGlu and [3 H]PteGlu are consistent with the lower binding capacity of FBP to [3 H]PteGlu in MTX-resistant *E. faecium* than in *E. faecium*/wild. The uptakes of 5-HCO-H₄PteGlu and PteGlu were more severely affected in *E. faecium*/MTX/5-HCO-H₄PteGlu than in *E. faecium*/MTX/PteGlu, suggesting an effect of the form of folate used in the media on the transport of folates. The decreased response of *E. faecium*/MTX/5-HCO-H₄PteGlu to PteGlu can, in part, be explained by this finding together with the lack of increase in DHFR activity and a relatively small increase in FAR activity in this mutant strain. It should be noted that the reduction in the capacity of FBP to bind to [3 H]PteGlu was not necessarily parallel to the uptake of [3 H]PteGlu in these MTX-resistant *E. faecium*. It may be that the FBP identified in this study is not only functioning as a transport protein, but also playing a role that has not been identified. A correlation between decreased MTX transport and reduced FBP binding capacity suggests a causal relationship in some cells (Saikawa *et al.*, 1993).

Our results indicate that wild-type as well as MTX-resistant *E. faecium* has the capability to synthesize MTXGlu from MTX, although this capability is much lower than that in *E. faecium*/wild. This suggests that the activities of folylpolyglutamate synthetase are lower in MTX-resistant strains than in wild-type *E. faecium* as reported by Jolivet *et al.* (1982). Furthermore, MTX-resistant *E. faecium* did not accumulate intracellular MTXGlu which is more toxic than MTX. MTXGlu

leached out in the media from MTX-resistant strains indicating that these mutant strains were able to efficiently eliminate this toxic compound. The amount of radioactivity found at the position of elution of 4-amino-4-deoxy-10-methylpteroic acid on Sephadex G-25 chromatography was similar in all three strains suggesting a similar ability to catabolize MTX to this degradation product.

Prescott & Affronti (1968) reported an increased folate conjugase activity in an MTX-resistant strain of *E. faecium* using yeast polyglutamates as substrate and suggested this finding as one of the mechanisms for *E. faecium* to develop MTX resistance. However, we did not find any activity of folate conjugase in MTX-resistant and wild-type *E. faecium* using synthetic PteGlu₃ as substrate. At the present time, it remains to be determined whether the discrepancy between our results and those of Prescott & Affronti (1968), is due to the difference in the substrates used for the assays. Nevertheless, the decreased MTXGlu levels found in MTX-resistant *E. faecium* could be caused by decreased synthesis of MTXGlu and/or increased degradation of MTXGlu in these resistant strains as compared to wild-type.

In summary, MTX resistance in *E. faecium* developed by a combination of several mechanisms including increased DHFR and FAR activities, decreased synthesis and retention of polyglutamyl MTX, decreased transport of MTX accompanied by reduced folate transport, and down-regulation of FBP. Among these, DHFR and FAR activities as well as folate transport were markedly affected by the form of folate supplied in the media during the development of MTX resistance. It may be important to use a reduced form of folate, more natural than oxidized PteGlu, in the media during the development of resistance for the study of the mechanisms of MTX resistance in cultured cells.

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