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# DEFECTIVE TRANSPORT OF AMINOPTERIN IN RELATION TO DEVELOPMENT OF RESISTANCE IN YOSHIDA SARCOMA CELLS

B. M. BRAGANCA, A. Y. DIVEKAR AND N. R. VAIDYA
*Biochemistry Division, Indian Cancer Research Centre, Parel, Bombay (India)*
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## SUMMARY

1. Biochemical changes responsible for cell resistance have been explored using two aminopterin-resistant cell lines ($YSR_1$, $YSR_2$) derived from a highly sensitive strain of Yoshida sarcoma ascites cells ($YSS_1$).

2. Both the resistant sublines had a high resistance index (>33) but only one of these ($YSR_1$) showed significant increase in dihydrofolic acid reductase activity as compared to the parent strain. The observed increased dihydrofolic acid reductase was an induced enzyme and was gradually lost by serial passage through animals in the absence of the drug.

3. Inhibition experiments using ouabain have demonstrated that the transport of aminopterin in the sensitive as well as the two resistant lines of Yoshida sarcoma cells involves the participation of ($Na^+$–$K^+$)-activated ATPase. The transport of aminopterin in one of the resistant types ($YSR_2$) was one-half that observed in the parent sensitive strain. Inability to detect defective aminopterin transport in the other resistant subline ($YSR_1$) was apparently due to its high reductase activity.

4. Evidence obtained using a cobra venom factor which selectively attacks certain cell membranes also indicates that both the resistant cell lines have an altered cell surface.

5. It is suggested that resistance to antifolics in Yoshida sarcoma tumour develops as a result of a selection of a cell species having a defective transport carrier system.

## INTRODUCTION

Biochemical changes responsible for development of cell resistance to folic acid analogues are not yet clearly understood. Considerable evidence shows that the development of resistance to antifolics in human leukaemia cells[1] and a variety of other tumours[2,3] is accompanied by an increased activity of dihydrofolic acid reductase (5,6,7,8-tetrahydrofolate:NAD(P) oxidoreductase, EC 1.5.1.3). This has led to the impression that the resistant cells are able to overcome the action of these

drugs by synthesising the target enzyme in larger quantities and thus lowering the inhibitory effect to a level which does not disturb the metabolism. However, ROBERTS, WODINSKY AND HALL[4] have demonstrated that the folic acid reductase activity of 26 different transplantable tumours could not give any measure of their susceptibility to amethopterin and HOSHINO *et al.*[5] observed that in the development of amethopterin-resistant clonal lines of L 1210 leukaemia, the resistance appeared much earlier than the increase in dihydrofolic acid reductase activity. It is apparent from such observations that greater activity of the target enzyme cannot always be correlated with the degree of resistance.

Results of a study reported here on an aminopterin-sensitive and two resistant cell sublines derived from the same tumour (Yoshida sarcoma) have shown that differences in the cell permeability have a direct bearing on development of resistance.

## MATERIALS AND METHODS

### *Development of various strains of Yoshida sarcoma cells*

*Yoshida sarcoma cells sensitive to aminopterin ($YSS_1$).* The tumour was regularly maintained in an ascitic form in Wistar rats (150–200 g) by biweekly transplantation of 0.8 ml fluid derived from the intraperitoneal cavity. Approx. $8 \cdot 10^7$ cells were injected *via* the intraperitoneal route.

TABLE I

DEVELOPMENT OF A RESISTANT SUBLINE OF YOSHIDA SARCOMA CELLS ($YSR_1$)

Each group consisted of 4 female rats. Controls were injected with 0.15 ml of 0.05 M phosphate buffer (pH 7.4) and died in about 5 days.

| *Generation No.* | *Dose of aminopterin (μg/kg body wt.)* | *Survival in days* |
|---|---|---|
| 1 | 10 | 5–6 |
| 2 | 20 | 5–7 |
| 3 | 30 | 9–10 |
| 4 | 40 | 12–14 |
| 5 | 50 | 6–7 |
| 6 | 60 | 11–13 |
| 7 | 70 | 7–9 |
| 8 | 80 | 5–6 |
| 9 | 90 | 7–9 |
| 10 | 100 | 6–9 |
| 11 | 100 | 4–5 |

*Resistant type of Yoshida sarcoma cells ($YSR_1$).* This type was developed according to the schedule given in Table I. Aminopterin was dissolved in 0.15 ml of 0.05 M phosphate buffer (pH 7.4) and injected (intraperitoneally) into rats 48 h after the inoculation of the tumour. It was found that after the 6th generation an additional dose of aminopterin (10 μg/kg body weight) given 15 min prior to the tumour inoculation caused resistance to develop faster. The cells became resistant after serial passage through 11 generations. This procedure was therefore routinely

adopted. The $YSR_1$ cells so obtained were passed through 2 generations of rats without aminopterin injections to free them from intracellular aminopterin, prior to their use in the experiments described in this study.

*Resistant type of Yoshida sarcoma cells ($YSR_2$).* This line was obtained by serial passage of $YSR_1$ cells through 7–8 generations of rats in the absence of aminopterin. The development of this line from $YSR_1$ cells was carried out independently 4 times. The biochemical properties described in this paper were studied for each set of experimental material.

*Preparation of Yoshida sarcoma cell suspension free of red blood cells*

The procedure followed was that described in the previous paper[6].

*Dihydrofolic acid reductase activity of the sensitive and resistant sublines*

*Preparation of cell extracts.* A cell suspension in Tyrode solution containing $6.25 \cdot 10^8$ cells was centrifuged at 1300 × *g* for 5 min. The cells were suspended in 0.15 M phosphate buffer (pH 5.5) so as to make 10 ml volume. For preparation of the extract, the suspension was sonicated for 1.5 min in a Raytheon sonicator and then centrifuged at 18 000 × *g* for 15 min. The supernatant was dialysed against 6 l of distilled water overnight. The small amount of sediment formed was removed by centrifuging again at 18 000 × *g* for 15 min. All the operations were carried out at 0 – 4°.

*Dihydrofolic acid reductase assay.* The dihydrofolic acid reductase activity of the tumour cell extracts was assayed by measuring the decrease of absorbance at 340 mμ due to NADPH reoxidation and reduction of dihydrofolic acid as described earlier[7]. The decrease of absorbance was measured for 5 min taking readings once every minute to check the linearity of the reaction. The decrease of absorbance was linear up to a decrease of 0.030 per min. The aliquots of the tumour cell extracts were adjusted to suit these conditions. $\Delta E$ used for calculating the amount of dihydrofolic acid reduced was 11 270 (see ref. 8).

*Pig heart extract.* This was prepared according to the method of OCHOA[9] to the first stage of purification and was used as a source of isocitric dihydrogenase for reducing $NADP^+$.

*Crystalline dihydrofolic acid.* This was prepared according to the method of BLAKLEY[10] and stored over $P_2O_5$ in the dark under vacuum. It was stable for 2–3 days.

*Protein determination.* Protein content of the tumour cell extracts was determined by the method of LOWRY *et al.*[11].

*Folic acid content of the sensitive and resistant sublines*

The folic acid content of the three cell types was determined by the method described in the previous paper[6].

*Aminopterin transport in sensitive and resistant sublines*

The procedure was the same as in the previous paper[6].

*Preparation and assay of cytotoxicity of cobra venom factor*

This was carried out as described by BRAGANCA, PATEL AND BADRINATH[12].

*Materials*

Materials were the same as those mentioned in the previous paper[6]. Amethopterin (sodium salt) was the product of American Cyanamid Co.

## RESULTS

*Resistance to aminopterin in 2 sublines of Yoshida sarcoma cells*

In order to explore the biochemical differences which may have a close bearing on the development of cell resistance in Yoshida sarcoma cells, two sublines of this tumour resistant to aminopterin were developed from a parent strain which was highly sensitive to destruction by this analogue. The resistant subline $YSR_1$ was produced by following the schedule described in MATERIALS AND METHODS. The subline $YSR_2$ was obtained by passage of $YSR_1$ cells through several generations of rats without aminopterin (see MATERIALS AND METHODS). Results given in Fig. 1 illustrate the effects of increasing doses of aminopterin on the survival time (days) of rats inoculated with the sensitive and the two resistant cell lines.

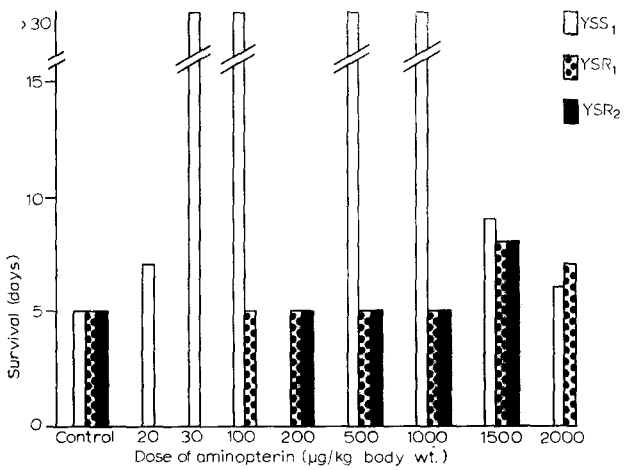



Fig. 1. Resistance to aminopterin in 2 sublines of Yoshida sarcoma cells ($YSR_1$ and $YSR_2$). For details see MATERIALS AND METHODS. Each column represents the average survival of 4 rats. Controls received 0.15 ml of 0.05 M phosphate buffer (pH 7.4).

A single dose of 30 $\mu$g aminopterin per kg body weight given intraperitoneally 48 h after the inoculation of the parent sensitive strain ($YSS_1$) completely protects all the animals against Yoshida sarcoma for an indefinite period (survival greater than 30 days). Injections of 1500 $\mu$g and more of aminopterin per kg body weight kill the animals in less than 10 days indicating that this level of aminopterin is toxic. In animals inoculated with the resistant subline $YSR_1$ or $YSR_2$, doses of 100 – 1000 $\mu$g aminopterin per kg body weight are unable to prevent growth of tumour and death occurs in less than 10 days. Resistance to higher doses of aminopterin could not be tested due to death resulting from toxic effects of aminopterin.

By expressing resistance index as:

$$RI = \frac{\text{Minimal dose of aminopterin required to prevent growth of tumour in all animals inoculated either with } YSR_1 \text{ or } YSR_2}{\text{Minimal dose of aminopterin required to prevent growth of tumour in all animals inoculated with } YSS_1 \text{ (parent strain).}}$$

The RI's for $YSR_1$ and $YSR_2$ are greater than 33.

*Dihydrofolic acid reductase activity of Yoshida sarcoma cells sensitive and resistant to aminopterin*

As cell resistance to antifolics[13] is generally associated with an increase in dihydrofolic acid reductase activity, the activity of this enzyme was determined in the sensitive and resistant sublines of Yoshida sarcoma cells ($YSS_1$; $YSR_1$; $YSR_2$). Results given in Table II show that the specific activity of dihydrofolic acid reductase

TABLE II

TOTAL SOLUBLE PROTEIN AND DIHYDROFOLIC ACID REDUCTASE ACTIVITY OF DIFFERENT SUBLINES OF YOSHIDA SARCOMA CELLS

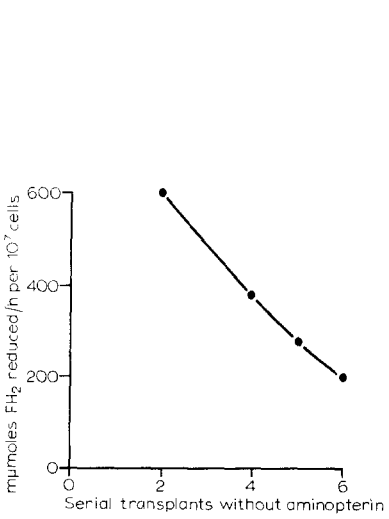
For assay of enzyme activity, see MATERIALS AND METHODS. The assay system contained: 2 μmoles sodium citrate; 15 μmoles of phosphate buffer (pH 5.5); 1 mg protein of pig heart extract; 0.16 μmole $NADP^+$; 125 μmoles $CaCl_2$; 3 μmoles 2,3-dimercapto-1-propanol; 0.1 μmole dihydrofolate; 0.1–0.3 ml tumour cell extract; and water to make 3 ml vol. Temp., 24°. The following controls were negligible: (1) All constituents except tumour cell extract. (2) All constituents except dihydrofolate. Figures in brackets indicate the number of experiments done.

| *Tumour subline* | *Soluble protein (μg/$10^7$ cells)* | *mμmoles dihydrofolic acid reduced per mg protein per h* | *mμmoles dihydrofolic acid reduced per $10^7$ cells* |
|---|---|---|---|
| $YSS_1$ | 419–457 (3) | 444– 586 (3) | 198–253 (3) |
| $YSR_1$ | 640–704 (5) | 907–1440 (5) | 607–989 (5) |
| $YSR_2$ | 409–575 (3) | 401– 446 (3) | 164–229 (3) |

is increased in the resistant strain $YSR_1$. It is 2.3 times higher than in the parent cells. As there is also an increase in the soluble protein in these cells, the total increase in the dihydrofolic acid reductase protein in $10^7$ cells ($YSR_1$) is 3.7 times higher than in cells of the sensitive strain. It is significant that there is no increase in the dihydrofolic acid reductase activity of the $YSR_2$ cells as compared to the parent sensitive cells ($YSS_1$). The total soluble protein in this subline is also the same as in the parent strain.

*Loss of induced dihydrofolic acid reductase activity in the absence of aminopterin*

Fig. 2 is a graph relating dihydrofolic acid reductase activity of $YSR_1$ cells to the number of serial transplant generations in the absence of aminopterin. It shows that the enzyme activity induced in these cells ($YSR_1$) during development of resistance, gradually decreases in the course of the serial passage of the resistant cells through rats in the absence of the antimetabolite. The enzyme activity in the resistant cells returned to the level present in the sensitive parent strain after the 6th generation.



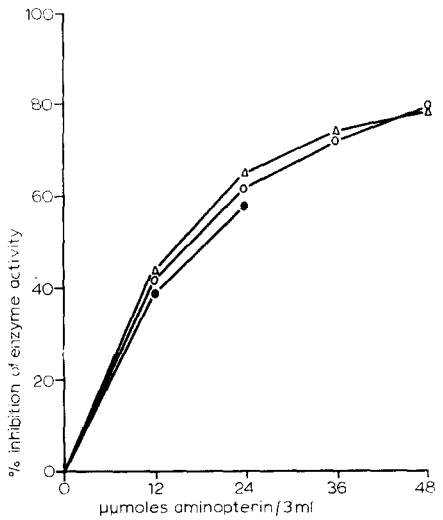




Fig. 2. Loss of induced dihydrofolic acid reductase activity in the absence of aminopterin. For experimental conditions see MATERIALS AND METHODS.

Fig. 3. Aminopterin inhibition of dihydrofolic acid reductase of the 3 sublines of Yoshida sarcoma cells. △—△, $YSS_1$; ○—○, $YSR_1$; ●—●, $YSR_2$. For experimental conditions see MATERIALS AND METHODS.

*Aminopterin inhibition of dihydrofolic acid reductase of the three sublines of Yoshida sarcoma cells*

In view of the significant differences observed in the specific activity of dihydrofolic acid reductase of the two resistant sublines, it was of interest to determine whether there were differences in the affinity of this enzyme for aminopterin in the three cell lines examined. In these experiments the inhibition produced by various concentrations of aminopterin on cell extracts having equivalent dihydrofolic acid reductase activity was measured. Fig. 3 relates the per cent inhibition of enzyme activity to the various concentrations of aminopterin used. The curves for the three cell lines are not significantly different.

*Transport of aminopterin in the sensitive and resistant sublines of Yoshida sarcoma cells*

As the experiments have shown that in one of the resistant sublines ($YSR_2$), cell resistance to aminopterin could not be associated with an increase in dihydrofolic acid reductase activity, the possibility of a defect in transport of aminopterin in the resistant sublines of Yoshida sarcoma cells was explored. Studies[6] employing the parent sensitive strain of Yoshida sarcoma cells have demonstrated that aminopterin is transported into these cells by an energy-dependent process against a concentration gradient. Results given in Fig. 4 illustrate the transport of aminopterin by the three sublines of Yoshida sarcoma cells for various concentrations of aminopterin (0.14–2.3 $\mu$M) in the medium. It is seen that for all concentrations examined, the transport of aminopterin in the resistant strain $YSR_2$ is half of that observed in the parent $YSS_1$ cells, whereas the aminopterin transport in the resistant $YSR_1$ subline is the same as in $YSS_1$ cells. Up to a level of 0.91 $\mu$M aminopterin, the rate

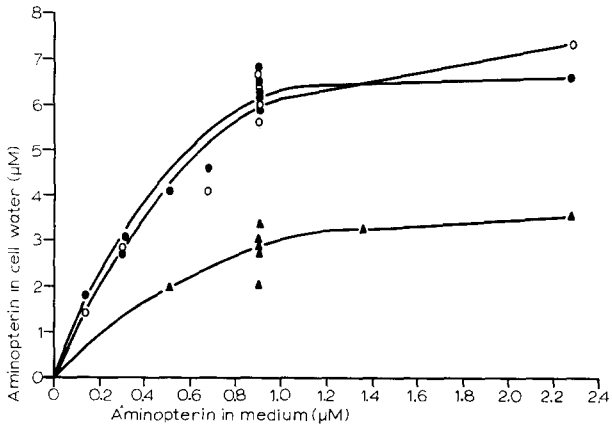



Fig. 4. Transport of aminopterin in the sensitive and resistant sublines of Yoshida sarcoma cells. ●—●, $YSS_1$; ○—○, $YSR_1$; ▲—▲, $YSR_2$. Number of Yoshida sarcoma cells, $1.25 \cdot 10^8$; total vol., 50 ml; time of incubation, 1 h; temp., 37°; pH 7.4.

of uptake is linearly proportional to the external concentration of the drug in all 3 cell lines. Above this concentration equilibrium is reached. At equilibrium approx. 0.6 mμmole of aminopterin is taken up by $YSS_1$ and $YSR_1$ cells ($10^8$) and 0.3 mμmoles by $YSR_2$ cells ($10^8$).

*Effect of ouabain on the transport of aminopterin in the sensitive and resistant sublines*

The effect of ouabain, an inhibitor of the energy-dependent transport system, was studied on the three types of cell lines. Results given in Table III show that the uptake is inhibited in every case by more than 50% in the presence of 10 μM ouabain.

TABLE III

EFFECT OF OUABAIN ON THE TRANSPORT OF AMINOPTERIN IN SENSITIVE AND RESISTANT SUBLINES

Experimental conditions were the same as described in Fig. 4. Aminopterin, 0.91 μM; ouabain, $10^{-5}$ M.

| *Tumour subline* | *Supplement added* | *Aminopterin in cell water (μM)* | *% inhibition* |
|---|---|---|---|
| $YSS_1$ | None | 6.16 | — |
| | Ouabain | 2.64 | 57 |
| $YSR_1$ | None | 6.26 | — |
| | Ouabain | 1.56 | 75 |
| $YSR_2$ | None | 2.93 | — |
| | Ouabain | — | 100 |

*Folic acid content of the sensitive and resistant sublines*

It is clear from Table IV that the folic acid content of $YSS_1$ and $YSR_1$ cells is about the same, whereas in $YSR_2$ cells it is 50% less.

*Membrane properties of the sensitive and resistant lines of Yoshida sarcoma cells*

Since significant differences have been observed in the transport of aminopterin

TABLE IV

FOLIC ACID CONTENT OF SENSITIVE AND RESISTANT SUBLINES OF YOSHIDA SARCOMA CELLS

For experimental conditions see MATERIALS AND METHODS. Figures in brackets show the number of experiments done.

| *Tumour subline* | *Folic acid (mμmole/10⁸ cells)* |
|---|---|
| $YSS_1$ | 0.50–0.80 (8) |
| $YSR_1$ | 0.45–0.71 (5) |
| $YSR_2$ | 0.06–0.35 (4) |

in the sensitive and resistant strain ($YSR_2$) of Yoshida sarcoma cells studied here, it was of interest to explore possible differences in the properties of the cell membranes of the three lines of cells. An approach to this problem was made using a cytotoxic protein isolated from cobra venom[12] which shows considerable selectivity in lysing Yoshida sarcoma cells as compared to a variety of other cell species. In these experiments the comparative effects of cobra venom factor on the cell surface of the three sublines were examined *in vitro* by determining the μg protein of the cytotoxic factor required to cause changes in the permeability of the cell to a dye (lissamine green) in 50% of the cells under standard conditions (see MATERIALS AND METHODS). It is clear from results given in Table V that the two resistant strains $YSR_1$ and $YSR_2$ show cross resistance to the cobra venom cytotoxin and required 2 – 3 times more of the cytotoxic protein to cause equivalent damage to their cell membranes.

TABLE V

EFFECT OF CYTOTOXIN FROM COBRA VENOM ON DIFFERENT SUBLINES OF YOSHIDA SARCOMA CELLS

For experimental conditions see MATERIALS AND METHODS.

| *Tumour subline* | *Cytotoxin μg protein/ml required to cause 50% lysis* |
|---|---|
| $YSS_1$ | 0.5–0.63 |
| $YSR_1$ | 1.0–1.66 |
| $YSR_2$ | 1.0–1.66 |

## DISCUSSION

It is evident from the data presented here that the two sublines of Yoshida sarcoma cells resistant to aminopterin had a high resistance index but only one ($YSR_1$) showed elevated reductase activity. It may be noted that the two aminopterin-resistant cell lines also showed cross resistance to amethopterin. The increased enzyme activity detected in the resistant subline was apparently induced during the passage of the cells in the presence of aminopterin. It was gradually lost during serial transplantations of the resistant tumour in the absence of the drug. At the end of 6 generations the activity reached the level present in the parent strain, whereas the resist-

ance remained unchanged. Experiments in which the cells were serially transplanted for 35 generations without the antimetabolite also showed that they were still resistant at the end of this period.

As SIROTNAK, DONATI AND HUTCHISON[14] have shown that dihydrofolic acid reductase protein from amethopterin-resistant *D. pneumoniae* had a decreased affinity for the inhibitor, such a possibility was tested in the present study. It is apparent from results given in Fig. 3 that the dihydrofolic acid reductase of the three sublines is equally susceptible to inhibition by aminopterin. Thus the resistance developed in $YSR_2$ cells whose dihydrofolic acid reductase was as low as in the sensitive strain could not be due to differences in the affinity of the enzyme for the inhibitor.

Exploration of other possible changes characteristic of the resistant lines has demonstrated significant differences in the transport of aminopterin in the sensitive and resistant strain ($YSR_2$). Detailed studies[6] on the kinetics of transport of aminopterin by Yoshida sarcoma cells sensitive to this drug ($YSS_1$) have clearly shown that the process involves an enzyme carrier system and that it is energy-dependent. Observations showing that ouabain inhibits the uptake of the drug in all 3 sublines indicate that the transport of aminopterin is associated with ($Na^+$–$K^+$)-activated ATPase. Most of the transported aminopterin was found[6] to be present in the cell sap where dihydrofolic acid reductase is located and part of it was bound to the enzyme. On the basis of such data the extent of aminopterin transported would be determined both by the activity of the enzyme carrier system present in the cell surface as well as by the pull resulting from the firm combination of aminopterin with dihydrofolic acid reductase inside the cells. Since the activity of this enzyme in the resistant subline ($YSR_2$) and the sensitive subline ($YSS_1$) is the same, it is logical to assume that the decreased transport of aminopterin observed in the former cells is caused by a defective carrier system. The failure to detect decreased transport in the other resistant subline ($YSR_1$) could be explained by the presence of the elevated dihydrofolic acid reductase activity of this line of cells, which exerted a stronger pull on the uptake of aminopterin, thus balancing the effects of the defective carrier system. This view implies that a defective carrier system is a common property of the cell surface of both the resistant cell lines, even though lowered transport may not always be apparent, as in the case of $YSR_1$ cells. Similar factors may explain the results of HALL, ROBERTS AND KESSEL[15] who found decreased transport of amethopterin in some of the amethopterin-resistant human lymphatic leukaemic cells, while in others an increased transport was observed. The authors assumed that the increased transport may be a reflection of elevated folic acid reductase activity. Thus in human leukaemia also, the resistance to amethopterin could be associated primarily with a defect in the transport of the analogue.

Further indication that both the resistant sublines of Yoshida sarcoma cells have an altered cell surface is provided by results from observations using the cytotoxin from cobra venom[12]. This cytotoxic protein which selectively attacks the cell surface of certain cells causing extensive morphological changes which finally lead to lysis, showed differential effects on the sensitive cells and the two resistant cell lines. It was found to be considerably more cytotoxic to the sensitive cells ($YSS_1$) whereas both the resistant strains were equally more resistant to the destructive effects of this protein.

From the overall evidence reported here it is conceivable that in Yoshida

sarcoma tumour, which consists of a heterogenous cell population, the resistance develops primarily by selection of a cell species which has deficient a carrier system for the transport of the analogues. The small amount of aminopterin which penetrates these cells possibly helps the induction of the target enzyme dihydrofolic acid reductase giving rise to a cell type characterised by $YSR_1$. In this subline both the induced enzyme level and the altered cell surface may contribute to its resistance. On removal of aminopterin however, the induced enzyme is gradually lost but the defective carrier system continues in the progeny thus resulting in a subline having the properties of $YSR_2$.

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