On the Radiosensitivity of the Radiation Induced Aminopterin Resistant Yeast Cells

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X線照射により得られたアミノプテリン抵抗酵母細胞の放射線感受性について

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酵母細胞を用いX線照射によりアミノプテリン抵抗細胞を得た．そこでTMP合成の過程の放射線障害と細胞死との関連の可能性を仮定して，このアミノプテリン抵抗細胞と原細胞を用い，両細胞の放射線感受性を比較検討し，TMP合成の過程が放射線のtargetの1つである可能性の証拠を追求した．

実験結果より，アミノプテリン抵抗細胞は原細胞より放射線感受性は低く，原細胞をTMPで前処置すると放射線感受性は低くなる．又excess AdRとexcess TMPは原細胞に対しては細胞増殖阻害効果があるのに反し，アミノプテリン抵抗細胞にはその効果を示さない．hydroxyureaとexcess dUMPは両細胞に対して細胞増殖阻害を示す．

これらの事実から，dUMPからTMPの過程は密接に放射線感受性と関連し，又targetの1つである可能性をもつと考えられる．

(I) Introduction

Various evidences have been accumulated to believe that DNA should be at least one of the target of radiation in cell death as well as in molecular damage.[5)8)] Since TMP synthesis should be one of the rate limiting factor of DNA synthesis and TMP was synthesized from dUMP, de novo, in case of necessity, even a slight disturbance caused by radiation might be related to the cell damage.[11)]

As it was well known that the path way from dUMP to TMP was mainly inhibited by aminopterin,[4)] comparative studies were carried out to find out correlation between radiosensitivity and this path way on original and aminopterin resistant cells. The aminopterin resistant cells were induced by irradiation in the preliminary experiment.

(II) Materials and Methods

Yeast cells (Saccharomyces Cerevisiae Sake) were cultured in Nägeli's solution (10 ml) in the test

tube kept in the temperature-control (28°C) incubator. The purified agar was added to Nägeli's solution in concentration of 2.0% to produce the plating medium on which the colony forming ability was studied.

Composition of Nägeli's solution

Ammonium tartrate	0.1 g
Potassium phosphate	0.1 g
Calcium chloride	0.02 g
Magnecium sulphate	0.02 g
Glucose	10.00 g
Aqua dest	100.00 ml

All irradiations were given with a 160 KVp X-ray apparatus. The doses were delivered at a dose rate of 2,500 R/min.

(III) EXPERIMENTS

(Experiment. A.) Radiation induced aminopterin resistant cells.

(Methods of experiment. A.)

The yeast cells harvested from the stationary phase which had been incubated in Nägeli's solution at temperature 28°C for several generations, were mingled enough by stirring with pipet. And these cells were put into the small plastic test tube and were irradiated with the dose of 37.5 KR. Immediately after the completion of irradiation, these cells were put into the test tube containing Nägeli's solution (10 ml) with or without aminopterin, the cell number being 100 cells/0.1 mm^3 of the solution and were incubated to get the growth curve.

As the control, not irradiated cells were incubated also in Nägeli with or without aminopterin. The comparison on the dose dependency was studied with cell growth curves. The cells incubated in aminopterin added medium, were pipetted on the plating medium containing aminopterin and the plate was incubated for 12 hours at 28°C to form colonies. The cells from the colony formed were returned to Nägeli's solution again and were incubated for some generations.

(Results of experiment. A.)

1) Non irradiated original cells were unable to grow in the aminopterin containing medium. (at final concentration 10^{-4} M.)

In Nägeli's medium without aminopterin, the growth of irradiated cells (37.5 KR) was some 24 hours slower than that of unirradiated cells. In aminopterin added medium (at final concentration 10^{-4} M.), irradiated cells (37.5 KR) could begin to grow from 7 days after incubation, contrary to unirradiated cells which never grow. (Fig. 1)

2) Dose dependency of the induction of aminopterin resistant cells was not observed in the range of 12.5 KR to 50 KR. (Fig. 2)

3) In the aminopterin minus medium, the growth curve of the aminopterin resistant cells was quite similar to that of the original cells, however in aminopterin added medium, the growth was delayed some 12 h. (Fig. 3)

(Experiment. B.) Comparison of radiosensitivities in colony formation of aminopterin resistant

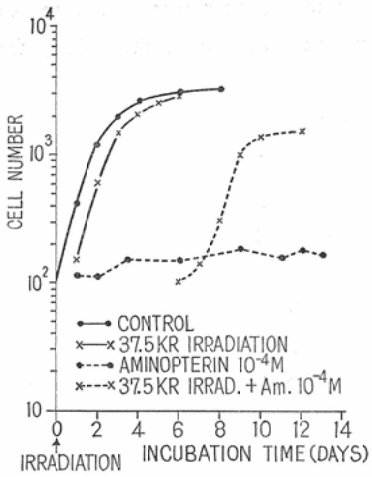

Fig. 1.

Growth curve
left solid line: control medium.
right brocken line: medium with aminopterin.
……×……: radiation induced aminopterin resistant cells.

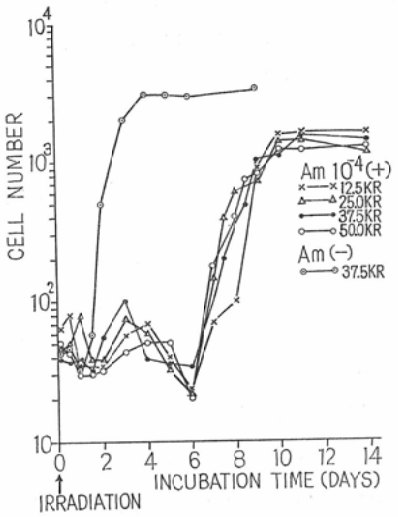

Fig. 2. Dose effect of X-irradiation on induction of aminopterin resistant cells growing in aminopterin added medium. (right group) and in control medium. (left)

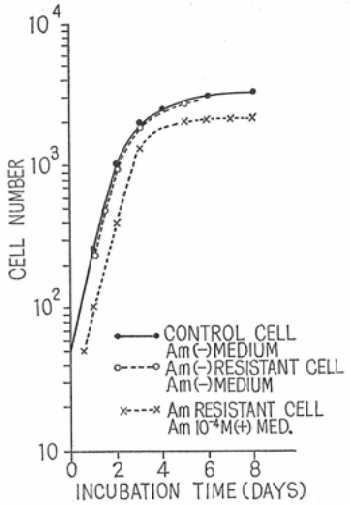

Fig. 3. Growth curves of both original (contral) and aminopterin resistant cells and that of the latter in aminopterin containing medium.

cells.

(Methods of experiment B.)

Both original or aminopterin resistant cells on growing phase were put in the small plastic tube respectively and were irradiated as described in experiment A. Immediately after irradiation (25, 37.5, 50, or 75 KR) both cells were diluted with saline and pipetted on the plating medium. After

the incubation for 15 h at 28°C, the cell death was determined by the colony forming ability to get the dose survival curve. Colonies containing less than 3 cells were scored as dead. 500 colonies were counted by microscope in each plate and the average survival % was calculated. To examine the role of TMP, TMP was added to the suspension of both cells in lag phase (16 or 24 hours after incubation) TMP final concentration being 10^{-5} M., 2 or 5 hours after addition of TMP, these cells were irradiated and incubated to get the does survival curve as above.

(Redults of experiment B.)

1) The dose survival curves of the cells harvested from growing phase both of original and of aminopterin resistant cells were shown in Fig. 4.

2) The dose survival curves of both cells treated with TMP were represented in Fig. 5.

3) It was represented in Fig. 6. that the growing curve of aminopterin resistant cells was quite similar to that of original cells, and aminopterin resistant cells were more radioresistant than original cells at every time of the growing phase. And the cells treated with TMP prior to irradiation increased the surviving fraction in original cells but not in aminopterin resistant cells.

(Experiment C.) Effects of DNA inhibitors on growth of aminopterin resistant cells.

(Methods of Experiment C.)

In my previous experiment,[15] it was proved that excess of AdR, of TMP and of dUMP or Hydroxyurea inhibited the growth of the original cells. These DNA inhibitors habing been known to act as a feed back inhibition on the path way from CMP to dCMP,[7)8)9)10)18)14)17)19)20)] were added in to Nägeli's solution (10 ml) in final concentration 2×10^{-3} M respectively. The aminopterin resistant cells in stationary phase were examined to obtain the growth curve in the medium in which

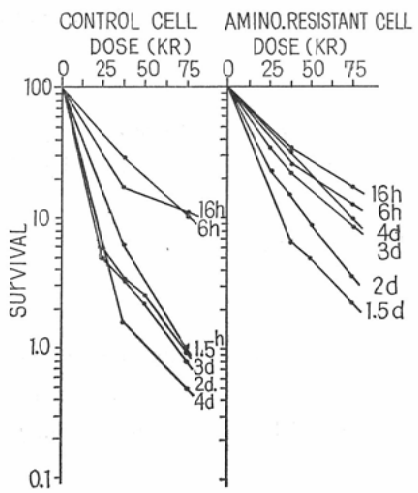

Fig. 4. Dose survival curves of both original and aminopterin resistant cells with respect to incubation time at irradiation.

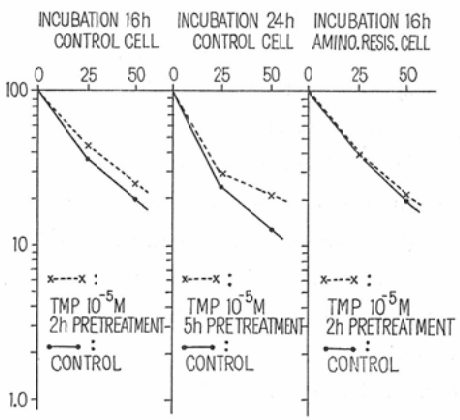

Fig. 5. Effects of TMP pretreatment on surviving fraction irradiated in lag phase. No effect on aminopterin resistant cells. (right)

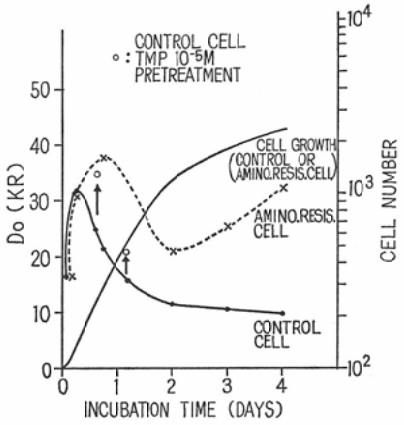

Fig. 6. Change of Do with respect to growing phase at irradiation. Growing pattern were simillar in both cells.
♀ indicats Do for TMP pretreated original cells.

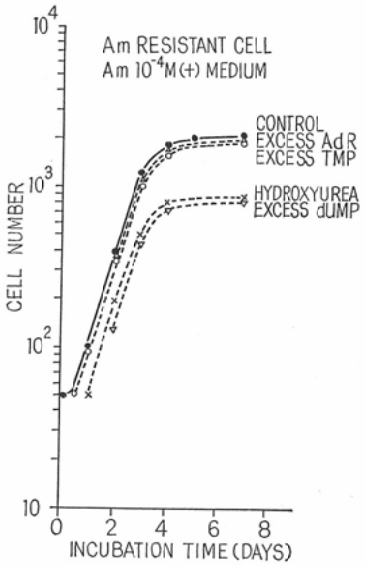

Fig. 7.
AdR: deoxyadenosine.
TMP: thymidine mononucleotide.
dUMP: deoxyuridine mononucleotide.
Effect of added deoxynucleoside or deoxynucleotide and hydroxyurea in high concentration (2×10^{-3}M) on aminopterin resistant cell growth.

aminopterin was added in addition to DNA inhibitor.

(Results of experiment C.)

The growth was not inhibited by excess of AdR as well as of TMP, contrary to that it was inhibited by excess of dUMP or by hydroxyurea. (Fig. 7.)

(IV) Results

1) The aminopterin resistant cells were induced by X-ray irradiation in yeast cells.

2) The aminopterin resistant cells were more radioresistant than original cells. And by TMP pretreatment, original cells became more radioresistant.

3) Hydroxyurea or excess of dUMP inhibited the cell growth of aminopterin resistant cells however excess of AdR or of TMP did not.

(V) Consideration and Conclusion

The aminopterin resistant cell was induced by irradiation, though dose dependency being undecided. Concidering the action of radiation, characteristics of the resistance seemed to imply repression of a certain enzymic activity as the result of DNA change which otherwise should take part in repression of the activity.

Regulatory mechanisms of TMP over-production or of unknown path way of TMP synthesis independent of folic acid contribution or of folic acid overproduction or of FH 2 reductace,[1-3)6)12)16)] were to be taken into account. Comparative studies of radiosensitivity were carried out on both

original and resistant cells. Owing to the similarity of both cells in growing pattern, it was able to compare the radiosensitivity which should vary with the growing phase of the cell. As the results, the aminopterin resistant cell was proved to be more radioresistant than the original cells.

From these experiments, it was suggested that the radiosensitivity was closely related to the quantity of TMP. Considering the folic acid dependent path way of TMP synthesis being inhibited by irradiation, TMP should be decreased in original cells and should not be influenced in resistant cells, another expriments which supported the difference of the two kinds of cell in TMP synthesis were that excess of AdR or TMP never inhibited the growth of aminopterin resistant cells contrary to that excess of deoxynucleotides except for dCMP inhibited the growth of original cells.[15]

Further, the original cells survived well by TMP addition to the medium prior to irradiation, contrary to the resistant cells being not influenced by TMP addition. My previous experiment[15] which indicated the enhancement of radiation effect by pretreatment with deoxynucleotides above mentioned also suggested the correlation between radiosensitivity and quantity of TMP. These evidences had a good reason to lead us to believe that path way from dUMP to TMP was closely related to the radiosensitivity or was of a possibility to be one of the target.

Summary

The aminopterin resistant cells were induced by radiation exposure in yeast cells.

Assuming that even a slight disturbance of TMP synthesis caused by radiation should be related to the cell damages, it was expected that a correlation should exist between radiation resistance and aminopterin resistance. In the present experiments, comparative studies of radiosensitivity were carried out on both original and resistant cells to evaluate this correlation in yeast cells, and it was proved that the aminopterin resistant cells were more radioresistant than original cells, and that original cells became more radioresistant by TMP pretreatment.

In these condition it was shown that though hydroxyurea or excess of dUMP inhibited the cell growth of aminopterin resistant cells, excess of AdR as well as of TMP never inhibited that.

From these evidences, it was proposed that path way from dUMP to TMP was closely related to the radiosensitivity.

References

1) Anton, H. and Nichol, C.A.: Some characteristics of A-Methopterinresistant strains of S. Faecalis. Proceedings of the American. Associ. for Can. Res. 2, 91, 1956.
2) Broquist, H.P. and Kohler, A.R.: Studies on the enzymatic formation of citrovorum factor by streptococcus faecalis. Journal of Biological Chemistry. 202, 59–66, 1953.
3) Bertino, T.R., Donohue, D.R., Gabrio, B.W., Silber, R., Alenty, A., Meyer, M. and Hoennekens, F.M.: Increased level of dihydrofolic reductase in leukocyte of patients treated with amithopterin. Nature 193, 140–142, 1962.
4) Chargaff, E. and Davidson, J.N.: The Nucleic Acids. Academic press inc. New York and London. 3, 456—469, 1960.
5) Freifelder, D.: Lethal changes in bacteriophage DNA produced by X-rays. Rad. Res. Suppl. 6, 80–96, 1966.
6) Friedkin, M., Crawford, E., Humphreys, S.R. and Goldin, A.: The association of increased dihydrofolate reductase with amethopterin resistant in mouse leukemia. Can Res. 22, 600–606, 1962.

7) Holnegren, A., Reichard, P. and Thelander, L.: Enzymatic synthesis of DNA. The effects fo ATP and dATP in the CDP reductase system from E. coli. Proc. Natl. Acad. Scien. 54, 830–836, 1965.
8) Kim, J.H., Kim, S.H. and Eidinoff, M.L.: Cell viability and nucleic acid metabolism after exposure of HeLa cells to excess thymidine and deoxyadenosine. Bioch. Parmacology. 14, 1821–1829, 1965.
9) Krakoff, I.H., Brown, N.C. and Reichard, P.: Inhibition of ribonucleoside diphosphate reductase by Hydroxyurea. Can. Res. 28, 1559–1565, 1968.
10) Kraloff, I.H., Brown, N.C. and Reichard, P.: Inhibition of ribonucleoside diphosphate reductase by hydroxyurea. Can. Res. 28, 1559–1565, 1968.
11) 気駕正己，他：放射線作用に対する核酸代謝の役割，日本医学放射線学会雑誌，26 (1966)，535—547.
12) Laboro, R., Maley, G.F. and Maley, F.: The effect of methotrexate on enzymes induced following partial hepatectomy. Can. Res. 29, 366, 1969.
13) Lambert, W.C. and Studjinski, G.P.: Recovery from prolonged growth induced in HeLa cells by high concentrations of thymidine. Can. Res. 27, 2364–2369, 1967.
14) Moore, E.C. and Hurlbert, R.B.: Regulation of mammalian deoxyribonucleotide biosynthesis by nucleotides as activators and inhibitors. J. Biol. Chem. 241, 4802–4809, 1966.
15) 宗近宏次：DNA 特に Deoxynucleotide 代謝阻害と放射線の増感作用について（特に Thymidine 及び Deoxcytidine を中心とする部分の阻害による），昭和医学会雑誌，29 (1969)，504—517.
16) Misra, D.K., Humphreys, S.R., Friedkin, M., Goldin, A. and Crawford, E.J.: Increased dihydrofolate reductase activity as a possible basis of drug resistance in leukemia. Nature. 189, 39–42, 1961.
17) Pfeiffer, S.E. and Tolmach, L.J.: Inhibition of DNA synthesis in HaLa cells by hydroxyurea. Can. Res. 27, 124–129, 1967.
18) Szybalski, W.: Molecular events resulting in radiation injury repair and sensitization of DNA. Rad. Res. Suppl. 7, 147–159, 1967.
19) Yarbro, J.W.: Further studies on the mechanism of action of hydroxyurea. Can. Res. 28, 1082–1087, 1968.
20) Yong, C.W., Schochetman, G., Hodas, S. and Balis, M.E.: Inhibition of DNA synthesis by hydroxyurea structure actibity relationships. Can. Res. 27, 535–540, 1967.