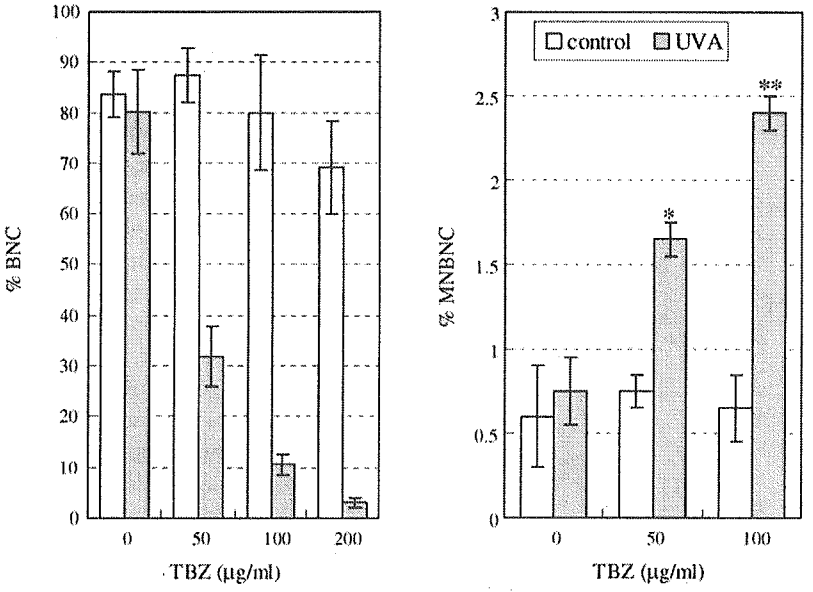


Fig. 3. Induction of micronuclei in WTK1 cells by pulse treatment with TBZ + UVA for 10 min in the cytochalasin-B/micronucleus test. Cell preparations were made 20 h after irradiation and micronuclei were counted in populations of 2000 binucleate cells. Open bar shows control cells without UVA-irradiation and shaded bar shows UVA-treated cells in both of the figures for % BNC (binuclear cells) and % MNBNC (micronucleated binuclear cells; $^{*}p < 0.05$, $^{**}p < 0.01$).

On the other hand, TBZ photomutagenicity in *E. coli* was observed following fluorescent illumination (15 W, 1860 lx, 10 min), probably due to small amounts of UVA and UVB emitted from the lamp (unpublished observation). UVA is the predominant source of radiant energy in sunlight. On the other hand, residue values of TBZ in citrus fruits were recommended not to exceed 10 ppm (FAO/WHO, 1973). We report here the induction of DNA damage and micronuclei by TBZ with UVA-irradiation in a human cell line. The photogenotoxic property of TBZ may be an important factor to consider when evaluating the risk to workers who spray TBZ on growing plants and on comported citrus fruits rather than residual intake from foods.

Acknowledgments

We thank Miriam Bloom for her critical reading of manuscript. This work was supported in part by Grants-in Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (15510061).

References

Barale, R., Scapoli, C., Meli, C., Casini, D., Minunni, M., Marrazzini, A., Loprieno, N., Barrai, I., 1993. Cytogenetic effects of benzimidazoles in mouse bone marrow. Mutat. Res. 300, 15–28.

Cheng, K.C., Cahill, D.S., Kasai, H., Nishimura, S., Loeb, L.A., 1992. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G→T and A→C substitutions. J. Biol. Chem. 267, 166–172.

FAO/WHO, 1973. 1972 evaluations of some pesticide residues in food. FAO/AGP/1972/M/9/1, WHO Pesticide Residues Series No. 2.

Fenech, M., 2000. The in vitro micronucleus technique. Mutat. Res. 455, 81–95.

Fenech, M., Morley, A.A., 1985. Measurement of micronuclei in lymphocytes. Mutat. Res. 147, 29–36.

Gatehouse, D., Haworth, S., Cebula, T., Gocke, E., Kier, L., Matsushima, T., Melcion, C., Nohmi, T., Ohta, T., Venitt, S., Zeiger, E., 1994. Recommendations for the performance of bacterial mutation assays. Mutat. Res. 312, 217–233.

Georgieva, V., Vachkova, R., Tzoneva, M., Kappas, A., 1990. Genotoxic activity of benomyl in different test systems. Environ. Mol. Mutagen. 16, 32–36.

Gocke, E., 1996. Review of the genotoxic properties of chlorpromazine and related phenothiazines. Mutat. Res. 366, 9–21.

Hayashi, M., Morita, T., Kodama, Y., Sofuni, T., Ishidate Jr., M., 1990. The micronucleus assay with mouse peripheral blood retic-

ulocytes using acridine orange-coated slides. Mutat. Res. 245, 245–249.

Kastenbaum, M.A., Bowman, K.O., 1970. Tables for determining the statistical significance of mutation frequencies. Mutat. Res. 9, 549–552.

Kato, T., Watanabe, M., Ohta, T., 1994. Induction of the SOS response and mutations by reactive oxygen-generating compounds in various *Escherichia coli* mutants defective in the *mutM*, *mutY* or *soxRS* loci. Mutagenesis 9, 245–251.

Kersten, B., Kasper, P., Brendler-Schwaab, S.Y., Muller, L., 2002. Use of the photo-micronucleus assay in Chinese hamster V79 cells to study photochemical genotoxicity. Mutat. Res. 519, 49–66.

Levy, J.A., Virolainen, M., Defendi, V., 1968. Human lymphoblastoid lines from lymph node and spleen. Cancer 22, 517–524.

Levin, D.E., Marnett, L.J., Ames, B.N., 1984. Spontaneous and mutagen-induced deletions: mechanistic studies in *Salmonella* tester strain TA102. Proc. Natl. Acad. Sci. U.S.A. 81, 4457–4461.

Levin, D.E., Ames, B.N., 1986. Classifying mutagens as to their specificity in causing the six possible transitions and transversions: a simple analysis using the *Salmonella* mutagenicity assay. Environ. Mutagen. 8, 9–28.

Lynch, A.M., Parry, J.M., 1993. The cytochalasin-B micronucleus/kinetochore assay in vitro: studies with 10 suspected aneugens. Mutat. Res. 287, 71–86.

Maron, D., Ames, B.N., 1983. Revised methods for the *Salmonella* mutagenicity test. Mutat. Res. 113, 173–215.

Marrazzini, A., Betti, C., Bernacchi, F., Barrai, I., Barale, R., 1994. Micronucleus test and metaphase analyses in mice exposed to known and suspected spindle poisons. Mutagenesis 9, 505–515.

McCarroll, N.E., Protze, I.A., Ioannou, Y., Frank Stack, H.F., Jackson, M.A., Waters, M.D., Dearfield, K.L., 2002. A survey of EPA/OPP and open literature on selected pesticide chemicals. III. Mutagenicity and carcinogenicity of benomyl and carbendazim. Mutat. Res. 512, 1–35.

Miller, J.H., 1992. A Short Course in Bacterial Genetics, A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria. Cold Spring Harbor Laboratory Press, New York.

Moriya, M., Ohta, T., Watanabe, K., Miyazawa, T., Kato, K., Shirasu, Y., 1983. Further mutagenicity studies on pesticides in bacterial reversion assay systems. Mutat. Res. 116, 185–216.

Mudry de Pargament, M., Labal de Vinuesa, M., Larripa, I., 1987. Mutagenic bioassay of certain pharmacological drugs: I. Thiabendazole (TBZ). Mutat. Res. 188, 1–6.

Oda, Y., Nakamura, S., Oki, I., Kato, T., Shinagawa, H., 1985. Evaluation of the new system (*umu*-test) for the detection of environmental mutagens and carcinogens. Mutat. Res. 147, 219–229.

Ohta, T., Nakamura, N., Moriya, M., Shirasu, Y., Kada, T., 1984. The SOS-function-inducing activity of chemical mutagens in *Escherichia coli*. Mutat. Res. 131, 101–109.

Ohta, T., Watanabe-Akanuma, M., Tokishita, S., Yamagata, H., 1999. Mutation spectra of chemical mutagens determined by Lac^+ reversion assay with *Escherichia coli* WP3101P-WP3106P tester strains. Mutat. Res. 440, 59–74.

Ohta, T., Tokishita, S., Tsunoi, R., Ohmae, S., Yamagata, H., 2002. Characterization of Trp^+ reversions in *E. coli* strain WP2*uvrA*. Mutagenesis 17, 313–316.

Sarrif, A.M., Bentley, K.S., Fu, L.J., O'Neil, R.M., Reynolds, V.L., Stahl, R.G., 1994. Evaluation of benomyl and carbendazim in the in vivo aneuploidy/micronucleus assay in BDF1 mouse bone marrow. Mutat. Res. 310, 143–149.

Sasaki, Y.F., Tsuda, S., Izumiyama, F., Nishidate, E., 1997. Detection of chemically induced DNA lesions in multiple mouse organs (liver, lung, spleen, kidney, and bone marrow) using the alkaline single cell gel electrophoresis (Comet) assay. Mutat. Res. 388, 33–44.

Sasaki, Y.F., Sekihashi, K., Izumiyama, F., Nishidate, E., Saga, A., Ishida, K., Tsuda, S., 2000. The comet assay with multiple mouse organs: comparison of comet assay results and carcinogenicity with 208 chemicals selected from the IARC Monographs and U.S. NTP carcinogenicity database. Crit. Rev. Toxicol. 30, 629–799.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191.

Stopper, H., Lutz, W.K., 2002. Induction of micronuclei in human cell lines and primary cells by combination treatment with γ-radiation and ethyl. Mutagenesis 17, 177–181.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen. 35, 206–221.

Van Hummelen, P., Elhajouji, A., Kirsch-Volders, M., 1995. Clastogenic and aneugenic effects of three benzimidazole derivatives in the in vitro micronucleus test using human lymphocytes. Mutagenesis 10, 23–29.

Venturini, S., Tamaro, M., Monti-Bragadin, C., Carlassare, F., 1981. Mutagenicity in *Salmonella typhimurium* of some angelicin derivatives proposed as new monofunctional agents for the photochemotherapy of psoriasis. Mutat. Res. 88, 17–22.

Watanabe-Akanuma, M., Ohta, T., Yamagata, H., 2003. Photomutagenicity of thiabendazole, a postharvest fungicide, in bacterial assays. Environ. Mol. Mutagen. 41, 92–98.

Xia, F., Wang, X., Wang, Y.H., Tsang, N.M., Yandell, D.W., Kelsey, K.T., Liber, H.L., 1995. Altered p53 status correlates with differences in sensitivity to radiation-induced mutation and apoptosis in two closely related human lymphoblast lines. Cancer Res. 55, 12–15.

遺伝毒性：DNA直接作用物質に閾値は存在するのか!?

祖父尼　俊雄[1*]，能美　健彦[2]，太田　敏博[3]，林　真[2]

[1]元国立医薬品食品衛生研究所変異遺伝部，[2]国立医薬品食品衛生研究所変異遺伝部
〒158-8501 東京都世田谷区上用賀1-18-1
[3]東京薬科大学生命科学部　〒192-0392 東京都八王子市堀之内1432-1

Genotoxicity: Is a threshold concept applicable to evaluate the mutagenic activity of DNA-targeting substances!?

Toshio Sofuni[1], Takehiko Nohmi[2], Toshihiro Ohta[3] and Makoto Hayashi[2]

[1]Ex-Director,
[2]Division of Genetics and Mutagenesis, National Institute of Health Sciences
1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan
[3]School of Life Science, Tokyo University of Pharmacy and Life Science
1432-1, Horinouchi, Hachioji, Tokyo 192-0392, Japan

Summary

The concept of a "biological threshold" is attracting interest as an evaluation criterion for the mutagenic activity of DNA-targeting mutagens. In this context, the concept is defined as "a concentration of a chemical which does not produce any damage through its inability to perform the necessary biochemical reactions, even though present at the target in finite amount". To clarify whether this criterion is indeed applicable to DNA-targeting mutagens, we re-evaluated the reverse mutation assay data using DNA repair-deficient bacterial strains, such as *S. typhimurium* strains lacking the O^6-methylguanine DNA methyltransferase genes (ada_{ST} and ogt_{ST}), the nucleotide excision repair gene (*uvrB*) or the 8-hydroxyguanine DNA glycosylase gene ($mutM_{ST}$), and *E. coli* strains lacking the nucleotide excision repair gene (*uvrA*). Mutagenic responses of 20 test chemicals including alkylating and non-alkylating agents were compared between the repair-deficient and their wild-type strains.

All the alkylating agents, such as MNNG, ENNG, EMS, ENU, DMN and DEN, exhibited more sensible mutagenic responses in strains YG7108 (Δada_{ST}, Δogt_{ST}) and YG7113 (same as YG7108 but containing the plasmid pKM101) than in the parental strains TA1535 and TA100 (same as TA1535 but containing the pKM101), respectively. Upon applying MNNG, YG7108 showed about 2−100 fold increase in the number of His^+ revertants above the spontaneous level over the range of 0.00025−0.25 μg/plate, whereas TA1535 did not show any significant increase in the number of His^+ revertants over the same dose range. On TA1535, an increasing tendency of the number of revertants was observed at 0.5 μg/plate or above. This indicates an approximate 2,000-fold difference at the mutagenic concentration level between the wild-type and the repair-deficient strains. Other alkylating agents also showed significant differences in mutagenic responses

* E-mail: toshi_sofu@yahoo.co.jp
Present address: 916-3 Kokuki, Shiraoka, Minami-Saitama, Saitama 349-0217, Japan
受付: 2005年4月5日　受理: 2005年4月5日

本稿は第33回日本環境変異原学会，第18回日本動物実験代替法学会合同学術会議，JEMS & JSAAE合同シンポジウム1「発がん性と遺伝毒性の閾値—リスクアセスメントにおける問題点—」で発表された。
This paper was presented to the JEMS & JSAAE combination symposium 1 "Threshold of carcinogenicity and genotoxicity —issues for risk assessment—" at the 33rd JEMS annual meeting and the 18th JSAAE annual meeting, 2004.

between YG7108 and TA1535, or between YG7113 and TA100 respectively, with some variations among test chemicals. On the other hand, non-alkylating agents, such as 4-NQO, AF-2, 2-NF and MX, did not show any differences in the dose-response relationships between YG7113 and TA100. When non-alkylating agents, such as 4-NQO, 2-NF and MX were applied to TA1535 (*ΔuvrB*), TA1538 (*ΔuvrB*) and WP2*uvrA* (*ΔuvrA*), clearly different mutagenic responses, i.e. about 30- to 60-fold, were observed between the repair-deficient and the parental strains (TA1975, TA1978 and WP2, respectively). 4-NQO showed different mutagenic responses between YG3002 ($\Delta mutM_{ST}$) and TA1975 (about 10-fold), though the application of other oxidative agents such as hydrogen peroxide resulted in less than 10-fold differences. The present results indicate that the wild-type strains having normal repair capacity show no gene mutation induction at the concentrations at which gene mutations are clearly induced in the repair-deficient strains through DNA damage. Thus, the present results suggest the existence of a "biological threshold" below which no mutagenic response is induced by DNA-targeting mutagenic substances.

Keywords: threshold, biological threshold, DNA-targeting mutagens, DNA repair -deficient strains, alkylating agents

緒　　言

遺伝毒性には閾値が存在しないという考え方がこれまで一般的に受け入れられてきたが，近年DNAを直接標的としない物質，例えば細胞分裂阻害剤やDNA合成阻害剤など，DNA以外の酵素や蛋白などへの影響によってもたらされる遺伝毒性には閾値が存在するとの考え方が国際的にも急速に受け入れられてきている(Elhajouji et al., 1995; 1997)．一方，DNAを直接標的とする物質には閾値が存在しないという考え方は依然として広く浸透しており，化学物質の安全性評価においてもこの考え方が支配的な状態にあるといえる．

閾値という概念にはいくつかの定義付けがなされている(Kirsch-Volders et al., 2000)が，その中に生物学的な閾値(biological threshold)という考え方が提唱されている．これはDNAを直接標的とする物質がDNAと直接作用する場がありながら，最終的な影響(例えば突然変異)の発現に必要とする全ての生物学的なプロセスが完遂できない(例えば修復メカニズムにより)低用量域が存在する，という考え方である．細菌を用いる復帰変異試験では*Salmonella typhimurium*(以下サルモネラ菌と略)のTA1535，TA1537，TA1538が用いられるが，これらはいずれもヌクレオチド除去修復欠損株(*ΔuvrB*)であり，それらの野生株であるTA1975，TA1977，TA1978に比べ変異原物質に対する感受性が高いことは一般に知られていることである．また，アルキル化DNA損傷を修復するO^6-methylguanine methyltransferase (MGT)を欠損した菌株が作製され，その野生株との比較から欠損株が野生株に比べてアルキル化剤に高感受性を示すことが認められている(Hakura et al., 1991; Yamada et al., 1995; 1997; 山田, 1999)．このようなDNA損傷修復欠損株を用いた研究では，多くの場合変異原物質の高感度検出や修復機構解明に視点が向けられ，必ずしも閾値の概念に結び付けようとするものではなかった．しかし，このようなDNA損傷修復欠損株と野生株の感受性の違いそのものが，上述の生物学的閾値の存在を意味しているものとして捉えるようになってきた(Sofuni et al., 2000)．DNAの酸化的損傷を修復する8-hydroxyguanine(8-OH-G)DNA glycosylaseを欠損する菌株が作製され，野生株との酸化的損傷に対する感受性が検討されており(Suzuki et al., 1997; Kim et al., 2004)，これらの菌株も含めて改めてこの問題について検討した結果を報告する．用いた菌株，被験物質，変異コロニー数のデータなどはここで引用した文献に基づいており，閾値の議論に必要な場合には，より低用量または高用量について同様の条件で追加実験を行っている．ヌクレオチド除去修復欠損株については本研究で得られた新たな実験結果を示している．

実験材料および方法

1) 使用菌株

細菌を用いる復帰突然変異試験に供した試験菌株をTable 1に示す．サルモネラ菌にはアルキル化DNA損傷修復酵素(O^6-methylguanine DNA methyltransferase：MGT)として2つの遺伝子ogt_{ST}とada_{ST}があり，これらを欠損した菌株YG7104(Δogt_{ST})とYG7108(Δogt_{ST}, Δada_{ST})およびその野生株TA1535，並びにTA1535株にpKM101プラスミドを導入したTA100とそのMGT欠損株YG7112(Δogt_{ST})とYG7113(Δogt_{ST}, Δada_{ST})を用いてアルキル化剤について突然変異誘発性を比較した．

非アルキル化剤については，ヌクレオチド除去修復欠損株を用いた．サルモネラ菌では，TA1535(*ΔuvrB*)とその野生株TA1975，TA1538(*ΔuvrB*)とその野生株TA1978，並びに大腸菌株ではWP2*uvrA*(*ΔuvrA*)とその野生株WP2との間で比較検討した．

酸化的DNA損傷については，サルモネラ菌の8-hydroxyguanine DNA glycosylaseの遺伝子欠損株を用いた．YG3001($\Delta mutM_{ST}$)とその野生株TA1535，YG3002($\Delta mutM_{ST}$)とその野生株TA1975，並びにYG3003

Table 1 Tester strains used in the present study

Strain	Description	Reference
Salmonella typhimurium		
TA1975	*hisG46, rfa*	Maron and Ames, 1983
TA1535	as TA1975, but *ΔuvrB*	Maron and Ames, 1983
YG7104	as TA1535, but Δogt_{ST}, Km^r	Yamada et al., 1995
YG7108	as TA1535, but Δada_{ST} and Δogt_{ST}, Km^r and Cm^r	Yamada et al., 1995
YG3001	as TA1535, but $\Delta mutM_{ST}$, Km^r	Suzuki et al, 1997
YG3002	as TA1975, but $\Delta mutM_{ST}$, Km^r	Suzuki et al, 1997
TA100	as TA1535, but harboring pKM101, Ap^r	McCann et al., 1975
YG7112	as TA100, but Δogt_{ST}, Cm^r	Yamada et al., 1997
YG7113	as TA100, but Δada_{ST} and Δogt_{ST}, Km^r and Cm^r	Yamada et al., 1997
TA102	*hisG428, rfa*, harboring pKM101, Ap^r	Suzuki et al, 1997
YG3003	as TA102, but $\Delta mutM_{ST}$, Km^r	Suzuki et al, 1997
TA1978	*hisD3052, rfa*	Maron and Ames, 1983
TA1538	as TA1978, but *ΔuvrB*	Maron and Ames, 1983
Escherichia coli		
WP2	*trpE65*	Gatehouse et al., 1994
WP2*uvrA*	as WP2, but *ΔuvrA*	Gatehouse et al., 1994

Km^r: resistant to kanamycin, Cm^r: resistant to chloramphenicol, Ap^r: resistant to ampicillin

Table 2 List of 20 test compounds

Abbreviation	Test compound	Source	Solvent
MNNG	*N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine	Dr. M. Nakadate, NIHS, Tokyo	Dimethyl sulfoxide
ENNG	*N*-Ethyl-*N'*-nitro-*N*-nitrosoguanidine	Dr. M. Nakadate, NIHS, Tokyo, and Sigma-Aldrich	Dimethyl sulfoxide
PNNG	*N*-Propyl-*N'*-nitro-*N*-nitrosoguanidine	Dr. M. Nakadate, NIHS, Tokyo	Dimethyl sulfoxide
BNNG	*N*-Butyl-*N'*-nitro-*N*-nitrosoguanidine	Dr. M. Nakadate, NIHS, Tokyo	Dimethyl sulfoxide
MMS	Methyl methanesulfonate	Tokyo Kasei Kogyo, Tokyo	Dimethyl sulfoxide
EMS	Ethyl methanesulfonate	Aldrich Chemical, Milwaukee	Dimethyl sulfoxide
MNU	*N*-Methyl-*N*-nitrosourea	Sigma Chemical Co., St. Louis	Dimethyl sulfoxide
ENU	*N*-Ethyl-*N*-nitrosourea	Sigma Chemical Co., St. Louis	Dimethyl sulfoxide
EDB	Ethylene dibromide	Wako Pure Chemical Industries, Osaka	Dimethyl sulfoxide
DMN	Dimethylnitrosamine	Wako Pure Chemical Industries, Osaka	Water
DEN	Diethylnitrosamine	Wako Pure Chemical Industries, Osaka	Water
4-NQO	4-Nitroquinoline 1-oxide	Tokyo Kasei Kogyo, Tokyo	Dimethyl sulfoxide or ethanol
AF-2	2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide (Furylfuramide)	Wako Pure Chemical Industries, Osaka	Dimethyl sulfoxide
2-NF	2-Nitrofluorene	Tokyo Kasei Kogyo, Tokyo, and Wako Pure Chemical Industries, Osaka	Dimethyl sulfoxide or ethanol
MX	3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone	Dr. N. Kinae, Shizuoka Prefectural Univ., and Wako Pure Chemical Industries, Osaka	Dimethyl sulfoxide
NPD	*p*-Nitro-*o*-phenylenediamine (1,2-diamino-4-nitrobenzene)	Wako Pure Chemical Industries, Osaka	Dimethyl sulfoxide
AZ	Sodium azide	Wako Pure Chemical Industries, Osaka	Water
HP	Hydrogen peroxide	Wako Pure Chemical Industries, Osaka	Water
MB (+light)	Methylene blue (+visible light)	Sigma Chemical Co., St. Louis	Water
NR (+light)	Neutral red (+visible light)	Junsei, Tokyo	Water

($\Delta mutM_{ST}$)とその野生株TA102をそれぞれ用いた．

2) 被験物質

DNAを直接標的とする代表的な物質として主にアルキル化剤を，また，その比較対照として塩基置換変異やフレームシフトを誘発する変異原物質や酸化的DNA損傷をもたらすものなど，20種類について検討した．それらの変異原物質の入手先と用いた溶媒の種類をTable 2に示した．

3) 試験方法

復帰突然変異試験は通常のプレインキュベーション法を用い，主に非代謝活性化法で行ったが，必要に応じてラット肝S9 mixによる代謝活性化法で行った．保存菌

Table 3 Number of His^+ revertants per plate in TA1535, YG7104 and YG7108 treated with MNNG in the absence of S9 mix

Experiment−1

Dose (μg/plate)	0			0.1			0.25			0.5			1		
Plate No.	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean
TA1535	12	10	11	11	7	9	4	9	7	21	14	18	361	489	425
YG7104	11	14	13	480	400	440	1344	1120	1232	1752	1920	1836	4000	2568	3284
YG7108	23	26	25	2704	2568	2636	3656	3968	3812	5520	5664	5592	4160	7024	5592

Experiment−2

Dose (μg/plate)	0			0.01			0.025			0.05			0.1			0.25			0.5		
Plate No.	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean
TA1535	6	8	7	8	6	7	6	2	4	2	4	3	4	4	4	8	2	5	18	34	26
YG7104	10	10	10	78	87	83	162	152	157	342	350	346	620	588	604	1601	1620	1611	5004	5508	5256
YG7108	37	46	42	824	962	893	1432	1346	1389	1230	1492	1361	1808	1846	1827	4008	5706	4857	5922	4888	5405

Dose (μg/plate)	1			2		
Plate No.	#1	#2	Mean	#1	#2	Mean
TA1535	860	986	923	2116	2126	2121
YG7104	5718	6066	5892	6218	8706	7462
YG7108	6006	6186	6096	7626	7326	7476

Experiment−3

Dose (μg/plate)	0			0.0001			0.00025			0.0005			0.001			0.0025			0.005		
Plate No.	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean
TA1535	7	5	6	10	11	11	7	ND	7	8	4	6	5	5	5	10	7	9	9	9	9
YG7104	22	20	21	27	27	27	37	25	31	24	23	24	36	29	33	38	39	39	71	76	74
YG7108	35	19	27	40	38	39	49	61	55	46	80	63	116	75	96	173	173	173	348	376	362

Dose (μg/plate)	0.01		
Plate No.	#1	#2	Mean
TA1535	8	9	9
YG7104	103	122	113
YG7108	452	488	470

(ND: No data)

液を解凍し，5～6 mLのニュートリエントブロスに5 μLの接種量で植え，37℃で15～16時間振盪培養した菌液を用いた．被験物質溶液 0.1 mLと0.1 M Na-リン酸緩衝液 0.5 mL(またはS9 mix)とテスト菌株の培養液0.1 mLを試験管に入れ，よく混合し37℃で20分間プレインキュベーションした．2 mLのトップアガーを加え，直ちに最少グルコース寒天プレート上に広げて固めた．プレートを37℃で48または72時間培養した後，His^+またはTrp^+復帰変異コロニー数を測定した．各用量毎に2～3枚のプレート(対照群は2～5枚)を用い，その平均値を求めた．

結　　果

1) MGT欠損株(Δogt_{ST}, Δada_{ST})による比較

代表的なアルキル化剤であるMNNGの結果をTable 3に示す．ここにはMGT欠損株であるYG7104(Δogt_{ST})とYG7108(Δogt_{ST}, Δada_{ST})，及びそれらの野生株TA1535についての3回の実験結果を示してある．TA1535の突然変異誘発に比べるとYG7104よりもYG7108の方がその差が大きいので，以後はYG7108のデータを用いて比較することにした．Fig. 1にYG7108とTA1535についての3回の実験結果をグラフで示してある．ここでは低用量域での反応を見やすくするため，X軸およびY軸共に対数で表示してある．YG7108では0.00025 μg/plateより明らかな変異コロニー数の増加がみられ，それ以後用量依存的に変異コロニー数が増加している．一方，TA1535の0.00025～0.25 μg/plateでは陰性対照と同等の値を示し，0.5 μg/plateでようやく変異コロニー数の増加傾向がみられ，1.0 μg/plateより顕著な増加がみられている．YG7108で突然変異が誘発される用量域(0.00025～0.25 μg/plate)において，TA1535ではogt_{ST}とada_{ST}の2つ遺伝子が正常に機能していることによって，突然変異の誘発が完全に抑えられていることが示されている．

ENNGについても同様の結果が得られている(Fig. 2)．YG7108では0.00025 μg/plateで変異コロニー数の増加傾向がみられ，それ以後用量依存的に変異コロニー数が増加している．一方，TA1535の0.01～0.5 μg/plateでは陰性対照と同等の値を示し，1.0 μg/plateで変異コロニー数の増加傾向がみられ，2.5 μg/plateで明らかな増加が

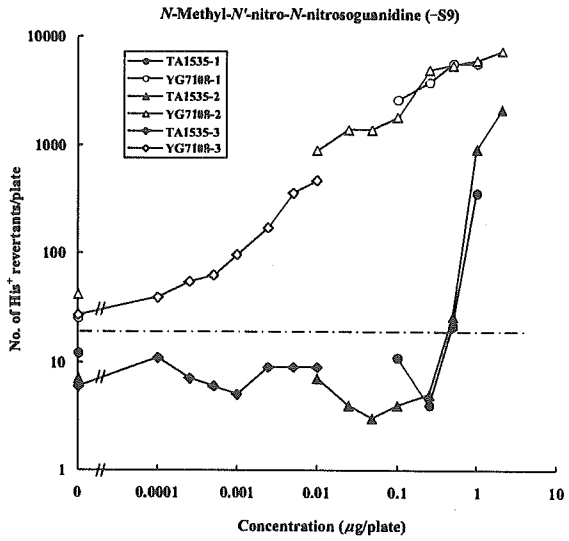


Fig. 1 Comparison of mutagenic responses of MNNG in TA1535 and YG7108 without S9 mix (3 experiment's data)

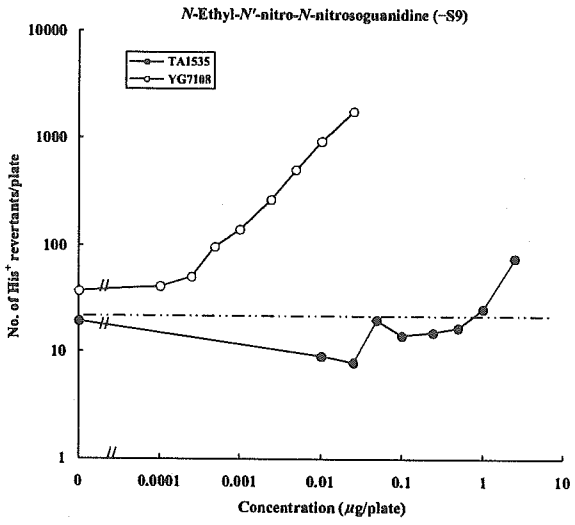


Fig. 2 Comparison of mutagenic responses of ENNG in TA1535 and YG7108 without S9 mix

Table 4 Number of His$^+$ revertants per plate in TA100, YG7112 and YG7113 treated with EMS in the absence of S9 mix

Experiment-1

Dose (μg/plate)	0			5			10			25			50			100			250		
Plate No.	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean
TA100	71	69	70	73	72	73	71	66	69	79	69	74	76	65	71	61	85	73	89	80	85
YG7112	78	70	74	240	216	228	328	320	324	800	880	840	1248	1232	1240	2304	1920	2112	2400	3040	2720
YG7113	70	79	75	216	236	226	448	456	452	1008	784	896	1280	1360	1320	2368	2784	2576	3040	3584	3312

Dose (μg/plate)	500			1000		
Plate No.	#1	#2	Mean	#1	#2	Mean
TA100	88	88	88	86	87	87
YG7112	3264	3456	3360	4160	4800	4480
YG7113	4224	3750	3987	4832	5824	5328

Experiment-2

Dose (μg/plate)	0			500			1000			2500			5000		
Plate No.	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean
TA100	82	60	71	78	63	71	83	75	79	170	168	169	1360	1120	1240
YG7113	120	129	125	4192	4224	4208	4012	4800	4406	5504	5600	5552	4800	5408	5104

認められている．2つの菌株で突然変異の誘発傾向のみられる用量で比較するとTA1535(1.0 μg/plate)はYG7108(0.00025 μg/plate)のおよそ4,000倍も高い用量となる．なお，MNNGでのこの差異はおよそ2,000倍である．

PNNGとBNNGについてもYG7108とTA1535との間で明らかな違いがみられているが，MNNGやENNG程の大きな違いではなく，突然変異の誘発傾向のみられる用量の差異はおよそ100倍程度と判断された(データを省略)．

EMSとMMSについてはTA100とそのMGT欠損株であるYG7112(Δogt_{ST})とYG7113(Δogt_{ST}, Δada_{ST})とで比較した．Table 4にEMSでの2回の実験結果を示す．1回目の実験でYG7113の方がYG7112より変異コロニーの誘発頻度が高い傾向にあるので，2回目の実験ではTA100とYG7113とで比較した．Fig. 3に両菌株での1回目と2回目の実験結果をグラフで示してある．YG7113では5.0 μg/plateで明らかな変異コロニー数の増加がみられ，それ以後用量依存的に変異コロニー数が増加している．一方，TA100では2,500 μg/plateから明らかな変異コロニー数の増加がみられており，その用量の差異は少なくとも500倍程になる．MMSではEMS程顕著な違いはみられておらず，およそ20倍程度の用量差にすぎなかった(データを省略)．

MNUおよびENUについてはTA1535とYG7108で比較を行い，ENUでは100倍を超える用量差があるものと判断され(Fig. 4)，MNUでも100倍に近い用量差があるものと判断された(データを省略)．EDBについて同

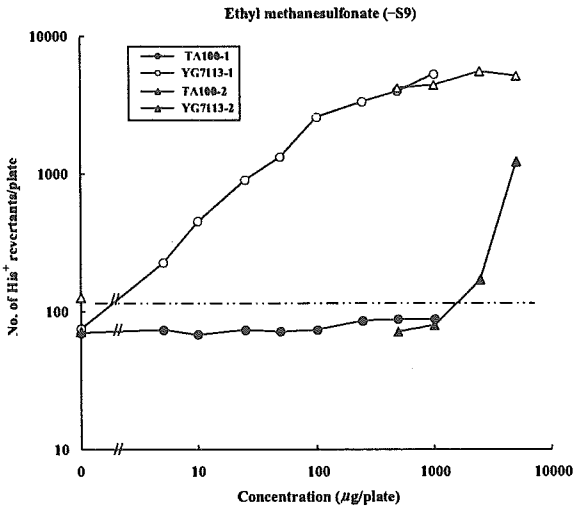


Fig. 3 Comparison of mutagenic responses of EMS in TA100 and YG7113 without S9 mix (2 experiment's data)

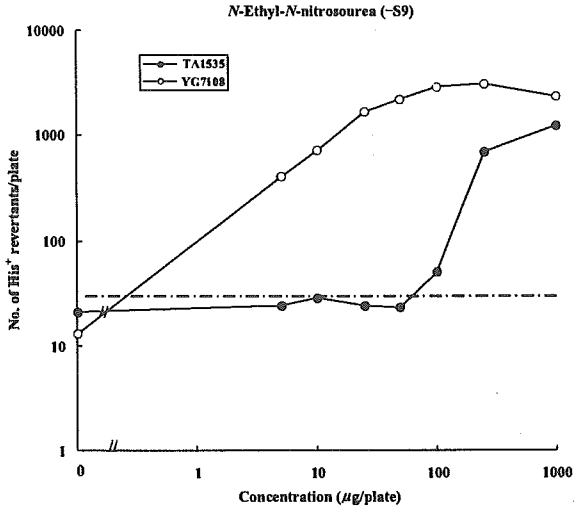


Fig. 4 Comparison of mutagenic responses of ENU in TA1535 and YG7108 without S9 mix

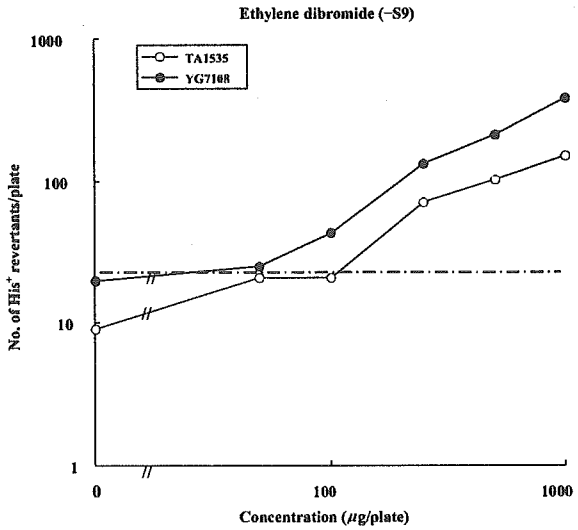


Fig. 5 Comparison of mutagenic responses of EDB in TA1535 and YG7108 without S9 mix

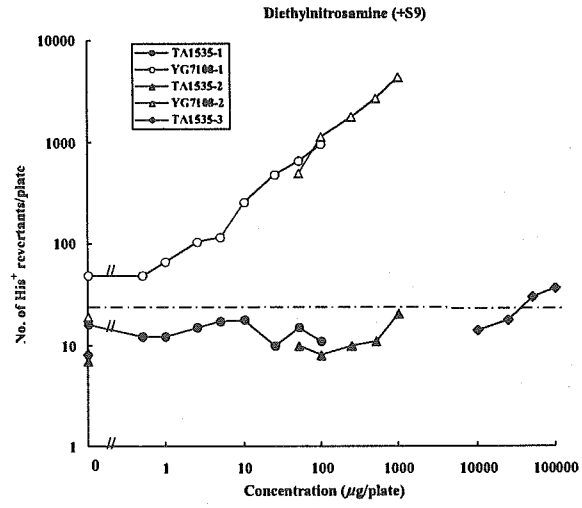


Fig. 6 Comparison of mutagenic responses of DEN in TA1535 and YG7108 with S9 mix (3 experiment's data)

様の比較を行ったが，両菌株での用量依存性には違いがあるものの，その用量差は10倍以下で明らかなものではなかった(Fig. 5)．

これまでは非代謝活性化法での実験であるが，代謝活性化法(ラットS9)を用いてDMNとDENついてTA1535とYG7108で比較検討した．DMNでは明らかに100倍を超える用量差があるものと判断された(データを省略)．DENのYG7108では2.5 μg/plateより変異コロニー数の明らかな増加がみられ，それ以後用量に依存して変異コロニー数が増加している(Fig. 6)．一方，YG1535では50,000 μg/plateという高用量で変異コロニー数の明らかな増加が認められている．これを直接比較すると20,000倍という極めて大きな違いになる．

非代謝活性化法を用いて4-NQO(Table 5)，AF-2(Fig. 7)，2-NFおよびMXについてTA100とYG7113とで比較したところ，両菌株の変異コロニー数の用量依存性はほとんど同じで，なんらの違いもみられなかった(2-NFとMXについてはデータを省略)．

2) ヌクレオチド除去修復欠損株(*uvrA* または *uvrB*)による比較

Ames試験に用いられているTA1535(*ΔuvrB*)およびTA1538(*ΔuvrB*)は除去修復の欠損株として知られており，それぞれの野生株であるTA1975とTA1978とで比較を行った．さらに大腸菌株WP2*uvrA*(*ΔuvrA*)とその野生株WP2との比較も行った．用いた被験物質は，

Table 5 Number of His$^+$ revertants per plate in TA100, YG7112, YG7113, TA1978, TA1538, TA1975 and YG3002 treated with 4-NQO in the absence of S9 mix

Dose (μg/plate)	0			0.01			0.025			0.05			0.1			0.25			0.5		
Plate No.	#1	#2	Av.	#1	#2	Av.	#1	#2	Av.	#1	#2	Av.	#1	#2	Av.	#1	#2	Av.	#1	#2	Av.
TA100	100	132	116	175	158	167	286	270	278	474	462	468	740	870	805	1338	1188	1263	1238	1194	1216
YG7112	184	154	169	212	226	219	324	284	304	462	454	458	742	684	713	1230	1234	1232	1618	1634	1626
YG7113	180	142	161	114	134	124	236	278	257	370	366	368	600	630	615	1192	1444	1318	1614	1526	1570

Dose (μg/plate)	0						0.0003				0.001				0.003			
Plate No.	#1	#2	#3	#4	#5	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean
TA1978	11	10	10	11	9	10	10	9	14	11	9	9	10	9	11	12	7	10
TA1538	12	12	8	15	9	11	10	11	11	11	12	12	13	12	8	16	9	11

Dose (μg/plate)	0.01				0.03				0.1				0.3				0.5			
Plate No.	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean
TA1978	12	7	13	11	8	12	10	10	10	12	14	12	13	13	7	11	15	11	10	12
TA1538	13	14	12	13	30	22	23	25	75	72	69	72	169	197	170	179	246	236	243	242

Dose (μg/plate)	1				2			
Plate No.	#1	#2	#3	Mean	#1	#2	#3	Mean
TA1978	10	12	17	13	21	22	25	23
TA1538	94	120	106	107	(21)	(25)	(28)	(25)

Dose (μg/plate)	0			0.5			1			2.5			5			10		
Plate No.	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean	#1	#2	Mean
TA1975	1	0	1	2	1	2	3	0	2	3	2	3	21	17	19	32	25	29
YG3002	1	2	2	8	7	8	8	21	15	92	84	88	630	600	615	630	563	597

(Parentheses indicate that apparent antibacterial activity was observed)

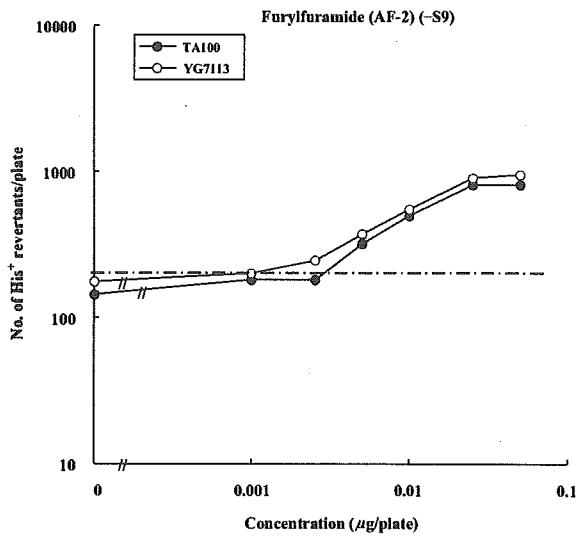


Fig. 7 Comparison of mutagenic responses of AF-2 in TA100 and YG7113 without S9 mix

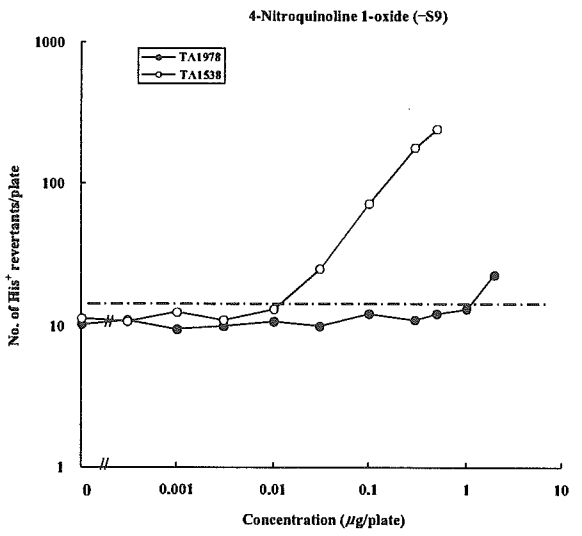


Fig. 8 Comparison of mutagenic responses of 4-NQO in TA1978 and TA1538 without S9 mix

TA100とYG7113との間で全く違いのみられなかった4-NQO, MX, 2-NFと, 新たにNaN3とNPDとを加えた5物質である. いずれも非代謝活性化法を用いた.

4-NQOについてTA1978とTA1538とで比較すると, TA1538では0.03 μg/plateより変異コロニー数の増加がみられ, 以後用量依存的に増加した. 一方, TA1978では2 μg/plateより変異コロニー数の明らかな増加がみられており, 60倍程の用量差があった(Table 5, Fig. 8). 同様の菌株の組み合わせで2-NFとNPDについて比較したところ, 前者ではその差異が少なくなり, およそ30倍程度であったが, 後者では両菌株の違いが大きくなり, 100倍程度の差がみられた(2-NFとNPDについてはデータを省略). MXについてはWP2とWP2*uvrA*とで比較したところ, WP2*uvrA*では0.03 μg/plateより明らかな変

Table 6 Number of Trp^+ revertants per plate in WP2 and WP2*uvr*A treated with MX in the absence of S9 mix

Dose (μg/plate)	0					0.0001				0.001				0.003				0.01			
Plate No.	#1	#2	#3	#4	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean
WP2	19	23	19	14	19	15	15	14	15	23	13	19	18	20	21	18	20	17	22	15	18
WP2*uvr*A	19	17	17	21	19	19	12	22	18	15	19	22	19	24	19	24	22	23	23	24	23

Dose (μg/plate)	0.03				0.1				0.3				1				3			
Plate No.	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean
WP2	11	21	20	17	20	23	14	19	22	25	25	24	31	42	44	39	99	102	88	96
WP2*uvr*A	81	85	81	82	222	202	228	217	852	755	843	817	796	812	836	815	(0)	(0)	(0)	(0)

Dose (μg/plate)	10				30			
Plate No.	#1	#2	#3	Mean	#1	#2	#3	Mean
WP2	319	286	295	300	427	448	574	483
WP2*uvr*A	(0)	(0)	(0)	(0)	ND	ND	ND	ND

(Parentheses indicate that apparent antibacterial activity was observed, ND: No data)

Table 7 Number of His^+ revertants per plate in TA1975 and TA1535 treated with AZ in the absence of S9 mix

Dose (μg/plate)	0					0.0001				0.0003				0.001				0.003			
Plate No.	#1	#2	#3	#4	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean
TA1975	1	1	0	2	1	2	0	1	1	ND	ND	ND	ND	1	0	2	1	ND	ND	ND	ND
TA1535	5	6	8	6	6	6	7	8	7	2	5	8	5	9	5	11	8	12	12	15	13

Dose (μg/plate)	0.01				0.03				0.1				0.3				1			
Plate No.	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean
TA1975	0	0	2	1	ND	ND	ND	ND	2	1	1	1	1	2	4	2	2	3	3	3
TA1535	12	13	16	14	25	26	27	26	66	83	87	79	247	246	243	245	607	670	594	624

Dose (μg/plate)	3				10				30				100			
Plate No.	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean
TA1975	7	4	4	5	16	19	20	18	37	35	26	33	62	49	56	56
TA1535	1278	1171	1319	1256	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

(ND: No data)

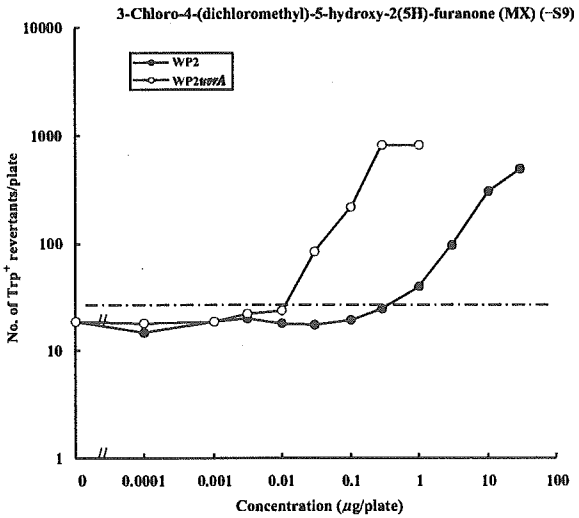


Fig. 9 Comparison of mutagenic responses of MX in WP2 and WP2*uvrA* without S9 mix

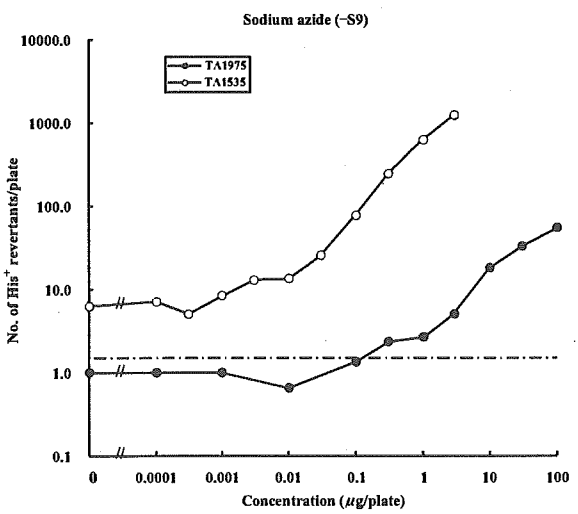


Fig. 10 Comparison of mutagenic responses of AZ in TA1975 and TA1535 without S9 mix

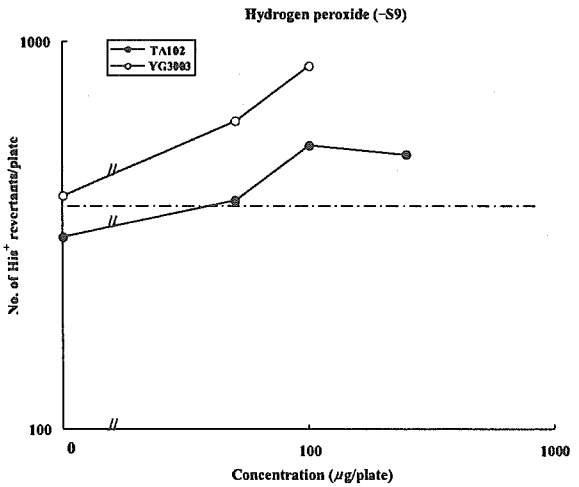


Fig. 11 Comparison of mutagenic responses of HP in TA102 and YG3003 without S9 mix

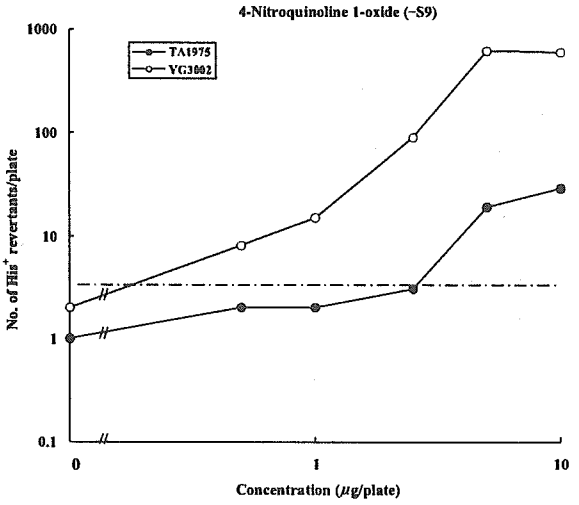


Fig. 12 Comparison of mutagenic responses of 4-NQO in TA1975 and YG3002 without S9 mix

異コロニー数の増加がみられ，WP2では1.0 μg/plateよりみられており，その用量差はおよそ30倍程度であった(Table 6, Fig. 9)．AZについてTA1975とTA1535とで比較すると，両菌株での用量差はNPDと同様に100倍程度であった(Table 7, Fig. 10)．なお，ENNGについてはTA1975とTA1535とで近似した用量依存性が得られたこと，およびTA1535とYG7104とでは突然変異の誘発に大きな違い(およそ3,000倍)のあることを別途確認している(データを省略)．

3) 酸化的損傷修復欠損株($mutM_{ST}$)による比較

DNA損傷の重要なものの1つである酸化的損傷について，YG3001($\Delta mutM_{ST}$)とその野生株TA1535，YG3002($\Delta mutM_{ST}$)とその野生株TA1975，YG3003($\Delta mutM_{ST}$)とその野生株TA102を用いて検討した．HPについては非代謝活性化法を用いてTA102とYG3003とで比較したが，その反応性に違いはあるものの，10倍以下であった(Fig. 11)．代謝活性化法を用いてMB＋可視光，NR＋可視光についてTA1535とYG3001およびTA1975とYG3002とでそれぞれ比較したが，いずれもHPと類似した反応を示した(データを省略)．一方，4-NQOについてTA1975とYG3002を用いて比較したところYG3002では0.5 μg/plateで変異コロニー数の増加がみられるのに対して，TA1975では5 μg/plateより変異コロニー数の増加がみられており，10倍程の用量差がみられた(Table 5, Fig. 12)．2-NFについてもTA1535とYG3001で比較したが，HPにおける反応と類似したものであった(データを省略)．

考　察

本研究結果のまとめをTable 8に示す．代表的なアルキル化剤であるMNNGやENNGでは，MGT欠損株YG7108で突然変異が誘発されている用量においても，その野生株TA1535では突然変異の誘発は全くみられず，両菌株間で突然変異の誘発傾向のみられる用量で比較すると，野生株では数千倍もの高い用量となる．このことは野生株においてはDNA損傷によって突然変異が生じる場が十分にありながら，その修復系の存在によって突然変異に固定されるまでの過程を完遂できない用量域，即ち生物学的な閾値が存在していることを示している．

PNNGとBNNGでも同様に明らかな違いがみられるが，MNNGやENNGに比べると，野生株と欠損株での違いの程度は低くなっているが，これは側鎖が大きくなるにつれてMGTによる修復が低下する一方で，他の修復系例えばヌクレオチド除去修復系が関与してくることなどが考えられる．ENUではMNNGやENNGとほぼ同様に野生株と欠損株とに違いがみられたが，MNUではPNNGやBNNGと同程度の違いがみられている．EDBでは，両菌株での用量依存性に差異はあるものの，その違いは10倍以下であり，突然変異の誘発にMGTが関与するDNA損傷の占める割合は少ないものと考えられる．

代謝活性化法を用いた場合にも同様の結果が得られている．DMNとDENは共に野生株と欠損株で比較すると100倍以上の差異がみられている．特に，DENの場合には直接的に比較すると20,000倍もの違いになっている．但し，野生株で変異コロニーが明らかに増加している用量は著しく高用量で，そのような高用量での代謝産

Table 8 Summary of the results in 20 test compounds compared with different tester strains

Deficient marker		ogt_{ST} and ada_{ST}		*uvrA* or *uvrB*			$mutM_{ST}$		
Deficient strain		YG7108	YG7113	TA1535	TA1538	WP2*uvrA*	YG3001	YG3002	YG3003
Wild strain		TA1535	TA100	TA1975	TA1978	WP2	TA1535	TA1975	TA102
MNNG	− S9	◎							
ENNG	− S9	◎		×					
PNNG	− S9	○							
BNNG	− S9	○							
MMS	− S9		○						
EMS	− S9		◎						
MNU	− S9	○							
ENU	− S9	◎							
EDB	− S9	△							
DMN	+ S9	◎							
DEN	+ S9	◎							
4-NQO	− S9		×		○			○	
AF-2	− S9		×						
2-NF	− S9		×		○		△		
MX	− S9		×			○			
NPD	− S9				○				
AZ	− S9			○					
HP	− S9								△
MB+light	+ S9						△		
NR+light	+ S9							△	

Differences of the lowest effective concentration between two tester strains were, ◎: more than 100-fold, ○: about 10- to 100-fold, △: less than 10-fold, ×: no difference

物の生成が低用量と同じ効率であるとは考えがたく，実際の差異はもっと小さいものと推測される．

TA1535で突然変異の誘発が認められない物質については，TA100(野生株)とYG7113(欠損株)とで比較したところ，EMSでは100倍以上，MMSでは10倍以上の違いがみられ，MGTが関与する生物学的な閾値が存在するものと考えられる．一方，4-NQO，AF-2，MXおよび2-NFではTA100とYG7113の変異コロニーの用量依存性に全く違いがみられず，これらの物質ではMGTが関与するようなDNA損傷が突然変異の誘発要因とはなっていないものと考えられる．

TA100とYG7113で全く違いのみられなかったもののうち，塩基置換変異を誘発するAZとMXについては，ヌクレオチド除去修復欠損株のTA1535(*ΔuvrB*)，WP2*uvrA*(*ΔuvrA*)とそれらの野生株TA1975，WP2株を用いて，フレームシフトを誘発する4-NQO，2-NF，NPDについてはTA1538(*ΔuvrB*)株とその野生株TA1978株を用いて比較したところ，いずれの場合も修復欠損株と野生株とには30～100倍程の差異がみられており，これらの変異原物質では除去修復系が生物学的閾値に寄与しているものと考えられる．

酸化的損傷については，$mutM_{ST}$欠損株YG3001(*Δ*$mutM_{ST}$)，YG3002(*Δ*$mutM_{ST}$)，YG3003(*Δ*$mutM_{ST}$)とそれらの野生株TA1535，TA1975，TA102を用いて比較したところ，HP，MB＋可視光，NR＋可視光ではいずれも差異はあるものの，その違いは10倍以下であった．酸化的損傷修復には$mutM_{ST}$に加えて$mutY_{ST}$も関与している．また，活性酸素などによるDNAの酸化的損傷は構造的に極めて多様であり，他の修復系が関与していることも考えられ，単一の修復酵素欠損株と野生株との比較ではその違いが明確には把握しにくい可能性が考えられる．一方，4-NQOではYG3002とTA1975との間に10倍以上の差異があり，$mutM_{ST}$による生物学的な閾値の存在を示唆している．4-NQOについては付加体形成に加えて8-OH-Gの生成が知られていることからも，酸化的損傷修復系が寄与する生物学的閾値が存在する可能性がある．今後より適切な実験系を組み立てることにより，酸化的損傷修復系による生物学的閾値の存在をより明確に確認できるものと考えられる．

チャイニーズ・ハムスター卵巣由来の細胞株CHOの亜株であるCHO-9ではMGT活性が検出限界以下であり，この細胞にヒトのMGTのcDNAおよび大腸菌の*ada*を導入したクローンが作られている(Kaina et al., 1993)．これらの細胞を用いて1 μM MNNGによるHPRT突然変異(6-TG抵抗性)を調べたところ，CHO-9では突然変異の誘発がみられているが，ヒトのMGTが発現している細胞では突然変異の誘発がほとんどみられていない．同様に2 mM ENUでの処理の場合は，ヒトのMGTを発現している細胞では突然変異の誘発はあるものの，CHO-9での誘発のおよそ1/5に低下していた．

20 μM MNNGによる染色体異常誘発についても検討しており，CHO-9では70%以上の細胞に染色体異常がみられたが，ヒトのMGTを発現している細胞では40%近くに低下し，さらに大腸菌*ada*導入細胞では染色体異常の誘発がほとんど抑えられていた(4%)．これらの結果はほ乳類培養細胞においても，細菌と同様の修復機構が存在し，それが遺伝子突然変異のみならず染色体異常にも生物学的閾値をもたらす可能性を示唆するものと考えられる．

ほ乳類では細菌の*MutM*遺伝子に相当するものとして*Ogg1*が知られており，この遺伝子のノックアウトマウスが作製され，突然変異検出系である*gpt*トランスジェニックマウスと交配させて，*gpt/Ogg1*$^{-/-}$マウスが作製されている．このマウスに腎発がん物質である臭素酸カリ($KBrO_3$) 2 g/Lを飲水で12週間投与したところ，*Ogg1*$^{-/-}$マウスの腎臓における8-OH-Gのレベルは*Ogg1*$^{+/+}$マウスの70倍程高く，*gpt*遺伝子の突然変異頻度も3倍程高まっていた(Arai et al., 2002)．この報告では1用量のみであるが，さらに低い用量段階をとると*Ogg1*$^{-/-}$マウスでは突然変異の誘発がみられる用量で，*Ogg1*$^{+/+}$マウスでは突然変異の誘発がみられない生物学的な閾値用量が見出される可能性が考えられる．

遺伝毒性発がん物質として知られているMeIQxについて*gpt* deltaトランスジェニックマウスを用いて突然変異の誘発が検討されている(Masumura et al., 2003)．3, 30, 300 ppmのMeIQxを12週間混餌で投与したところ，肝臓での*gpt*の突然変異頻度は30, 300 ppmで有意な増加が認められたが，3 ppmでは陰性対照と同等の突然変異頻度で有意な差異は認められなかった．さらに変異体について塩基配列を解析したところ，30, 300 ppmではMeIQx特有のG:C→T:Aトランスバージョンの増加がみられたが，3 ppmでは陰性対照にみられるものと同等であった．一方，MeIQxについてのラットを用いた実験では0.01 ppmよりDNA付加体の増加がみられるとの報告がある(Fukushima, 1999)．これらの報告は実験動物でも，DNA損傷をもたらすような用量においても種々の修復系によって突然変異に固定されない生物学的な閾値が存在することを示唆している．

変異原物質の閾値の問題についてはかなり以前からたびたび議論が繰り返されてきているが，その中でも日本環境変異原学会が1984年に開催した国際シンポジウムは注目に値する(Tazima et al., 1984; 黒田, 1984)．ここでは生殖細胞を経由して次世代に現れる突然変異と投与効果(単回高用量vs.複数回低用量)や代謝活性化による影響の問題等に加えて，DNA修復能の影響も論議されている．DNA修復と細胞交替についての論文(Kondo et al., 1984)では，ショウジョウバエの除去修復欠損系(*mus201/mus201*)とその野生系とで紫外線照射による眼色突然変異の誘発を比較しており，欠損系で突然変異の誘発がみられる照射量の40倍の照射量においても，野生系では明らかな突然変異の誘発はみられないことが報告されている．この論文では大腸菌でのデータに加えて，種々のヒトがん細胞株のうちO^6-methylguanine DNA methyltransferase欠損株(Mer$^-$)と野生株(Mer$^+$)とでMNNGによる姉妹染色分体交換(sister chromatid exchange: SCE)の誘発を比較した論文(Day et al., 1980)をも引用し(欠損株でSCEが誘発される用量において，野生株ではSCEの誘発がみられず，その10倍以上の用量からようやくSCEの誘発がみられている)，生物的障害をもたらすDNA損傷を「完全に」取り除ける高効率な修復系が発現している場合には閾値効果が認められるものと論じている．

このシンポジウム開催の基点となったと考えられる論文がシンポジウム開催2年前に報告されている(田島, 1982)．この論文では閾値の可能性が考えられるものとして4つの場合を提示し，その1つがDNA損傷の完全な修復としている．また，変異原物質の投与から生物的障害が成立するまでにさまざまな段階があるのに，どの段階なのかの区別がないままに閾値の議論がされていることの問題点を指摘しており，今日なおそれに近い状況にあるといえよう．さらに，自然発生レベルの生物的障害に妨げられて低用量での閾値を明確にできないような場合においても，「risk-benefit balanceを良く考えて」(原文引用)判定すべきで，「こうした実際的方法を非科学的と非難することは必ずしも当たらないと思う」(原文引用)とも述べており，ヒトへのリスク評価に向けて閾値を適用すべきスタンスを示している．20年以上も前に既にこのような見解が示されていることに改めて注目すると共に，科学的な実証をベースにしながらも，具体的に社会的な貢献ができる道筋を明確にしていくことが重要であると考える．

生物学的閾値とほぼ同様の考え方が本邦において既に提示されている(田島, 1980)．この論文では，標的に物質が到達していながらも無作用域がある場合を真の閾値とし，標的に物質が到達していないことによる無作用域を見かけ上の閾値と定義している．前者については農薬(シメトリン)による染色体異常誘発の例を引用し，ある用量以下では染色体異常の誘発がみられないが，そのような用量でもSCEの誘発がみられていることから，物質が標的に到達していながらも染色体異常の誘発がみられない，つまり染色体異常において真の閾値の存在を示す例として述べている．さらに，防かび剤(methyl-2-benzimidazoryl carbamate)による小核誘発の例が紹介されており，腹腔内投与と経口投与による小核誘発の違いが血中濃度の違いに依存している，つまり腹腔内投与ではあるレベル以上に血中濃度が上がらずしかも小核の誘発がないが，経口投与ではその倍以上に血中濃度が上がり小核の誘発がみられている．これは標的が暴露されて

いながらも，ある一定量を超えないと影響がみられないことから，真の閾値の存在する例として引用している．本稿の緒言で示した生物学的閾値の引用論文(Kirsch-Volders et al., 2000)には "Real(or biological)threshold" と記載されているのだが，「真(real)」という言葉の意味が伝わりにくいと判断し，あえて本稿では「生物学的(biological)閾値」という用語で通した．しかし，標的に物質が到達している状況が確認できる用量で，生物的障害がみられない事例については，「真の閾値」という用語の使用が普及していくことになるものと思われる．

結　　語

DNAを直接標的とする変異原物質について細菌を用いる復帰突然変異試験でDNA修復能の有無による突然変異誘発の違いを検討した．DNA修復欠損株で明らかに変異コロニーを誘発する用量，つまりDNA損傷により突然変異が生じうる用量においても，DNA修復能をもつ野生株では変異コロニーの誘発が認められておらず，生物学的な閾値が存在すると考えられる．細菌のみならず他の生物種においてもそのようなメカニズムの存在を示唆する研究成果が散在しており，生物学的な閾値が普遍的な考え方としてヒトのリスク評価に応用できるようになることを期待している．そのため今後特に実験動物等でのこのような事象の確認の積み重ねが重要であると考えられる．

謝　　辞

MGT欠損菌株を用いて追加の実験をして頂きました国立医薬品食品衛生研究所変異遺伝部，松井恵子技術補助員，並びに貴重な助言を頂きました同部，本間正充室長、山田雅巳主任研究官および同所安全情報部，森田健主任研究官に深謝致します．本研究に協力して頂きました日本環境変異原学会臨時委員会「食品および食品添加物等に関する遺伝毒性の検出・評価・解釈」の委員，並びに厚生労働科学研究費「既存添加物等における遺伝毒性評価のための戦略構築に関する研究」の班員に感謝致します．

本研究の一部は平成16年度厚生労働科学研究費補助金(食品の安全性高度化推進研究事業)「既存添加物等における遺伝毒性評価のための戦略構築に関する研究(H15−食品−009)」により実施した．

参考文献

Arai, T., V.P. Kelly, O. Minowa, T. Noda and S. Nishimura (2002) High accumulation of oxidant DNA damage, 8-hydoroxyguanine, in *Mmh/Ogg1* deficient mice by chronic oxidative stress, Carcinogenesis, 23, 2005-2010.

Elhajouji, A., P. Van Hummelen and M. Kirsch-Volders (1995) Indications for a threshold of chemically induced aneuploidy in vitro in human lymphocytes, Environ. Mol. Mutagen., 26, 292-304.

Elhajouji, A., F. Tibaldi and M. Kirsch-Volders (1997) Indication for thresholds of chromosome non-disjunction versus chromosome lagging induced by spindle inhibitors in vitro in human lymphocytes, Mutagenesis, 12, 133-140.

Day, R.S. III, C.H.J. Ziolkowski, D.A. Scudiero, S.A. Meyer, A.S. Lubiniecki, A.J. Girardi, S.M. Galloway and G.D. Bynum (1980) Defective repair of alkylated DNA by human tumour and SV40-transformed human cell strains, Nature, 288, 724-727.

Fukushima, S. (1999) Low-dose carcinogenicity of a heterocyclic amine, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline: relevance to risk assessment, Cancer Lett., 143, 157-159.

Gatehouse, D., S. Haworth, T. Cebula, E. Gocke, L. Kier, T. Matsushima, C. Melcion, T. Nomi, T. Ohta, S. Venitt and E. Zeiger (1994) Recommendations for the performance of bacterial mutation assays, Mutat. Res., 312, 217-233.

Hakura, A., K. Morimoto, T. Sofuni and T. Nohmi (1991) Cloning and characterization of the *Salmonella typhimurium* ada gene, which encodes O^6-methylguanine-DNA methyltransferase, J. Bacteriol., 173, 3663-3672.

Kaina, B., C. Fritz and T. Coquerelle (1993) Contribution of O^6-alkylguanine and N-alkylpurines to the formation of sister chromatid exchanges, chromosomal aberrations, and gene mutations: New insights gained from studies of genetically engineered mammalian cell lines, Environ. Mol. Mutagen., 22, 283-292.

Kim, S.-R., K. Matsui, M. Yamada, T. Kohno, H. Kasai, J. Yokota and T. Nohmi (2004) Suppression of chemically induced and spontaneously occurring oxidative mutagenesis by three alleles of human *OGG1* gene encoding 8-hydroxyguanine DNA glycosylase, Mutat. Res., 554, 365-374.

Kirsch-Volders, M., M. Aardema and A. Elhajouji (2000) Concepts of threshold in mutagenesis and carcinogenesis, Mutat. Res., 464, 3-11.

Kondo, S., H. Ryo, K. Fujikawa and A. Fukunaga (1984) The threshold effect in mutagenesis by radiation and chemicals in relation to DNA repair and cell replacement, In: Y. Tazima, S. Kondo and Y. Kuroda (Eds), Problems of Threshold in Chemical Mutagenesis, The Environmental Mutagen Society of Japan, Shizuoka, pp. 125-131.

黒田行昭 (1984) 環境化学物質の遺伝的影響の投与量—効果関係，とくに閾値の問題に関する国際シンポジウム，トキシコロジーフォーラム，7，655-659.

McCann, J., N.E. Spingarn, J. Kobori and B.N. Ames (1975) Detection of carcinogens as mutagens: Bacterial tester strains with R factor plasmids, Proc. Natl. Acad. Sci. USA, 72, 979-983.

Maron, D.M. and B.N. Ames (1983) Revised methods for the Salmonella mutagenicity test, Mutat. Res., 113, 173-215.

Masumura, K., M. Horiguchi, A. Nishikawa, T. Umemura, K. Kanki, Y. Kanke and T. Nohmi (2003) Low dose genotoxicity of 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) in *gpt* delta transgenic mice, Mutat. Res., 541, 91-102.

Sofuni, T., M. Hayashi, T. Nohmi, A. Matsuoka, M. Yamada and E. Kamata (2000) Semi-quantitative evaluation of genotoxic activity of chemical substances and evidence for a biological threshold of genotoxic activity, Mutat. Res., 464, 97-104.

Suzuki, M., K. Matsui, M. Yamada, H. Kasai, T. Sofuni and T. Nohmi (1997) Construction of mutants of *Salmonella typhimurium* deficient in 8-hydroxyguanine DNA glycosylase and their sensitivities to oxidative mutagens and nitro compounds, Mutat. Res., 393, 233-246.

Tazima, Y., S. Kondo and Y. Kuroda (1984) Problems of threshold in chemical mutagenesis: Proceedings of the symposium on dose-response relationship for genetic effects of environmental chemicals, Keidanren Kaikan, Tokyo, May 7-9, 1984, The Environmental Mutagen Society of Japan, Shizuoka.

田島弥太郎(1980)環境変異原研究当面の課題，環境変異原研究，2，2-9.

田島弥太郎(1982)化学変異原における閾値の問題，環境変異原研究，4，32-36.

Yamada, M., B. Sedgwick, T. Sofuni and T. Nohmi (1995) Construction and characterization of mutans of *Salmonella typhimurium* deficient in DNA repair of O^6-methylguanine, J. Bacteriol., 177, 1511-1519.

Yamada, M., K. Matsui, T. Sofuni and T. Nohmi (1997) New tester strains of *Salmonella typhimurium* lacking O^6-methylguanine DNA methyltransferases and highly sensitive to mutagenic alkylating agents, Mutat. Res., 381, 15-24.

山田雅巳 (1999) 遺伝子工学的手法を用いたアルキル化剤高感受性サルモネラ試験菌株の作製とその応用，環境変異原研究，21，123-130.

DOI: 10.1002/cmdc.200600008

A PEG-Based Biocompatible Block Catiomer with High Buffering Capacity for the Construction of Polyplex Micelles Showing Efficient Gene Transfer toward Primary Cells

Naoki Kanayama,[a, d] Shigeto Fukushima,[a, c] Nobuhiro Nishiyama,*[b] Keiji Itaka,[b] Woo-Dong Jang,[a] Kanjiro Miyata,[a] Yuichi Yamasaki,[a, d] Ung-il Chung,[b] and Kazunori Kataoka*[a, b, d]

Nonviral gene vectors from synthetic catiomers (polyplexes) are a promising alternative to viral vectors. In particular, many recent efforts have been devoted to the construction of biocompatible polyplexes for in vivo nonviral gene therapy. A promising approach in this regard is the use of poly(ethylene glycol) (PEG)-based block catiomers, which form a nanoscaled core–shell polyplex with biocompatible PEG palisades. In this study, a series of PEG-based block catiomers with different amine functionalities were newly prepared by a simple and affordable synthetic procedure based on an aminolysis reaction, and their utility as gene carriers was investigated. This study revealed that the block catiomers carrying the ethylenediamine unit at the side chain are capable of efficient and less toxic transfection even toward primary cells, highlighting critical structural factors of the cationic units in the construction of polyplex-type gene vectors. Moreover, the availability of the polyplex micelle for transfection with primary osteoblasts will facilitate its use for bone regeneration in vivo mediated by nonviral gene transfection.

Introduction

Gene therapy is a promising approach for the treatment of genetic and intractable diseases and for tissue engineering; however, its success still strongly depends on the development of useful gene vectors.[1] Recently, nonviral vectors based on the complexation of plasmid DNA (pDNA) with synthetic cationic polymers (catiomers) have attracted a great deal of attention as an alternative to viral vectors.[2–4] These vectors, the so-called polyplexes, are aimed toward both efficient transfection and decreased cytotoxicity.[5,6] In particular, there has recently been a strong impetus toward engineering the constituent catiomers to construct biocompatible polyplexes suitable for gene delivery in vivo.[2,5] A promising approach in this regard is the block copolymerization of catiomers with poly(ethylene glycol) (PEG) to obtain PEG-*block*-catiomers, as they spontaneously associate with pDNA to form polyplex micelles at the sub-100-nm scale with a dense and hydrophilic PEG palisade surrounding the polyplex core (Figure 1).[7–10] These polyplex micelles with PEG palisades showed high colloidal stability under physiological conditions and afforded appreciable levels of reporter-gene expression to various cell lines even after preincubation in a serum-containing medium.[7] Notably, the polyplex micelles demonstrated longevity in blood circulation,[11] offering the possibility of their use in systemic gene delivery. Nevertheless, a major obstacle to the successful application of this biocompatible nonviral vector system remains: the limited transfection efficacy toward primary cells.

Herein, we report a novel approach to obtain PEG-*block*-catiomers with remarkably high transfecting activity even toward primary cells, which are known to be sensitive to the toxicity induced by conventional polyplexes. The synthetic strategy for novel block catiomers is based on our unprecedented finding that the flanking benzyl ester groups of poly(β-benzyl L-aspartate) (PBLA) can undergo a quantitative aminolysis reaction with various polyamine compounds under mild anhydrous

[a] *Dr. N. Kanayama, S. Fukushima, Dr. W.-D. Jang, K. Miyata, Dr. Y. Yamasaki, Prof. Dr. K. Kataoka*
Department of Materials Engineering
Graduate School of Engineering
The University of Tokyo
Tokyo 113-8656 (Japan)
Fax: (+81) 3-5841-7139
E-mail: kataoka@bmw.t.u-tokyo.ac.jp

[b] *Dr. N. Nishiyama, Dr. K. Itaka, Prof. Dr. U.-i. Chung, Prof. Dr. K. Kataoka*
Center for Disease Biology and Integrative Medicine
Graduate School of Medicine
The University of Tokyo, Tokyo 113-0033 (Japan)
Fax: (+81) 3-5841-7139
E-mail: nishiyama@bmw.t.u-tokyo.ac.jp

[c] *S. Fukushima*
R&D Division, Pharmaceuticals Group
Nippon Kayaku Co., Ltd. (Japan)

[d] *Dr. N. Kanayama, Dr. Y. Yamasaki, Prof. Dr. K. Kataoka*
Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST) (Japan)

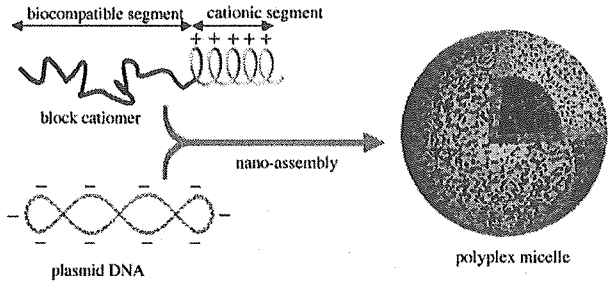
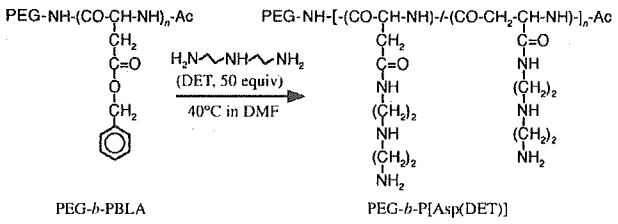


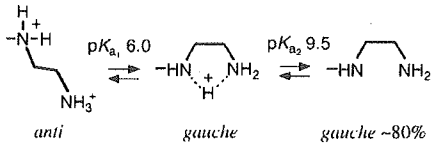
Figure 1. Formation of polyplex micelles through the electrostatic interaction between block catiomers and plasmid DNA.

conditions at 40 °C, thus allowing the preparation of cationic polyaspartamides with different amine functionalities, yet with the same molecular weight and distribution (Scheme 1). In particular, this study is focused on the unique properties of the



Scheme 1. Synthesis of PEG-*b*-P[Asp(DET)] block catiomer through the aminolysis of PEG-*b*-PBLA. DMF = *N*,*N*-dimethylformamide.

ethylenediamine unit integrated into the polyaspartamide side chain. Notably, ethylenediamine is known to undergo a clear two-step protonation with a distinctive *gauche–anti* conformational transition as depicted in Scheme 2,[12] and is thus expected to provide an effective buffering function in the acidic en-



Scheme 2. Two-step protonation of the ethylenediamine unit with a distinctive *gauche–anti* conformational transition.

dosomal compartment (pH 5). It has been suggested that catiomers with a low pK_a value such as polyethylenimine could buffer endosomal acidification and cause an increase in osmotic pressure in the endosome, leading to the disruption of the endosomal membrane to facilitate polyplex transport into the cytoplasm (the so-called proton sponge effect[13]). Indeed, PEG-*block*-polyaspartamide with an ethylenediamine unit at the side chain (PEG-*b*-P[Asp(DET)]) showed a remarkably high transfection efficacy to various cancer cells as well as mouse primary osteoblast cells. Importantly, this block catiomer was found to have remarkably low toxicity, facilitating its use for in vivo gene therapy.

Results and Discussion

PEG-*b*-polyaspartamide carrying the *N*-(2-aminoethyl)aminoethyl group $-(CH_2)_2NH(CH_2)_2NH_2$ as the side chain (PEG-*b*-P[Asp(DET)]) was prepared by the aminolysis of PEG-*b*-PBLA in dry DMF at 40 °C for 24 h in the presence of a molar excess (50 equiv relative to benzyl groups) of diethylenetriamine (DET) (Scheme 1). The ^{1}H NMR spectrum of PEG-*b*-P[Asp(DET)] is shown in Figure 2, and the ^{13}C NMR spectrum is available in

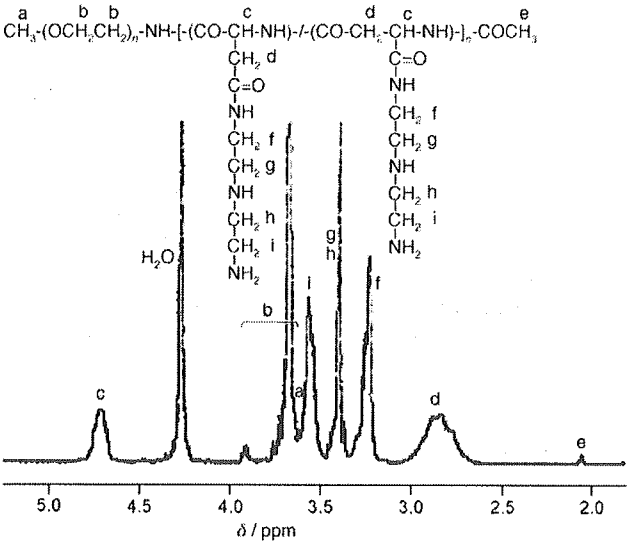


Figure 2. ^{1}H NMR spectrum of PEG-*b*-P[Asp(DET)] (solvent: D_2O, T = 80 °C); the polymer is in a salt form.

the Supporting Information. These data indicate that the aminolysis of the PBLA benzyl groups proceeded in a selective manner to the primary amine moiety of DET. Also, comparison of the integration ratio of the proton peaks (b and f–i) in Figure 2 reveals quantitative introduction of DET into the side chain of PBLA, and a unimodal molecular weight distribution of the obtained polymer was revealed by size-exclusion chromatography (SEC) measurement (Supporting Information). These results suggest a minimal occurrence of inter- or intrapolymer cross-linking by DET during aminolysis. Note that the peaks from the carbonyl and methylene groups of the aspartamide units in the ^{13}C NMR spectrum (Figure S1, Supporting Information) are split into two peaks, suggesting that the aminolysis of PBLA might induce intramolecular isomerization of the aspartamide units to form β-aspartamide. Figure 3 shows the time course of the aminolysis reaction of PBLA with DET, which was evaluated from the change in the ratio of the proton peak integration (f over b) in the ^{1}H NMR spectrum (Figure 2). This result indicates a fast and quantitative aminolysis of PBLA, which is in marked contrast to the lack of aminolysis with poly(γ-benzyl L-glutamate) (PBLG) under the same re-

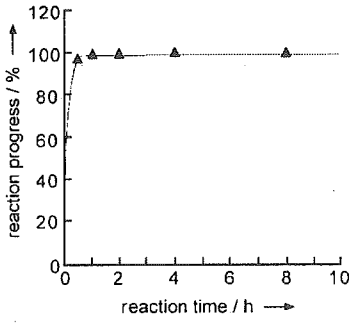


Figure 3. Time course of the aminolysis of PEG-*b*-PBLA with DET in DMF at 40 °C. The reaction progress was estimated from the change in the ratio of the proton peak integration (f over b) in the ^{1}H NMR spectrum (Figure 2).

action conditions (data not shown), highlighting a unique mechanism involved in the aminolysis of PBLA under mild conditions. Presumably, the amide groups of the main chain interact with the carbonyl group of the side chain, which may facilitate the aminolysis reaction.[14] The details of the mechanism of this unique aminolysis reaction are now under investigation in our research group and will be reported elsewhere.

The pH-dependent protonation of PEG-*b*-P[Asp(DET)] in media containing 150 mM NaCl was evaluated by potentiometric titration. The α/pH curve shown in Figure 4 clearly indicates

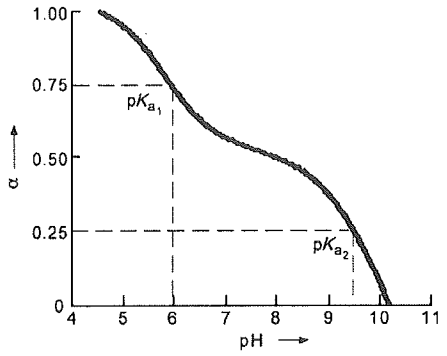


Figure 4. Degree of protonation (α) as a function of pH (α/pH curve) for the PEG-*b*-P[Asp(DET)] block catiomer (150 mM NaCl, aq, 25 °C).

the two-step protonation behavior of PEG-*b*-P[Asp(DET)], which is attributable to the two-step protonation of the ethylenediamine moiety with a distinctive *gauche--anti* conformational transition as indicated in Scheme 2. The two distinct pK_a values of the ethylenediamine moiety in the side chain of polyaspartamide were determined to be 6.0 and 9.5. Notably, this group remains nearly 100% populated by the mono-protonated state (*gauche* form) at pH 7.4, and is capable of exerting a substantial buffering effect in the pH range down to 5.0, at which point the equilibrium shifts to the di-protonated state (*anti* form) (Scheme 2).

The polyplex micelle was prepared by mixing solutions of PEG-*b*-P[Asp(DET)] and pDNA in various ratios of *N/P*, for which *N* is the total number of amine groups in the block catiomer and *P* represents the number of phosphate units in the pDNA. The formation of the polyplex, which accompanies pDNA condensation, was followed by an ethidium bromide (EtBr) dye-exclusion assay at different pH values. As shown in Figure 5, the

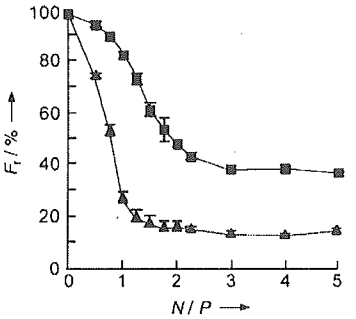


Figure 5. Effect of pH (▲: pH 5.0, ■: pH 7.4) on the relative fluorescence intensity (F_r) of EtBr in solution with pDNA and PEG-*b*-P[Asp(DET)] at various *N/P* ratios.

fluorescence intensity of EtBr decreases with an increase in the *N/P* ratio. At pH 5.0, the fluorescence of EtBr levels off at *N/P* = 1, which is consistent with approximately 95% protonation of the ethylenediamine unit, as expected from the α/pH curve in Figure 4. On the other hand, at pH 7.4, substantial quenching occurred at $N/P \approx 2.0$, which is consistent with the hypothesis that the mono-protonated form of the ethylenediamine unit in PEG-*b*-P[Asp(DET)] might be maintained even inside the polyplex. It is possible that the stabilized *gauche* conformation (Scheme 2) of the mono-protonated form may prevent the ethylenediamine unit from further protonation facilitated by the zipper effect or the local electrostatic field effect in the complexation process with anionic pDNA at pH 7.4.[15] The cumulant diameters and ζ potentials of the polyplexes prepared at different *N/P* ratios are shown in Figure 6. The cumulant diameters of the polyplex micelles were determined to be 70–90 nm throughout the range of the examined *N/P* ratios of 1–20, and the ζ potentials of the polyplexes increased with *N/P* ratios and leveled off at $N/P = 2$ (Figure 6). At $N/P > 2$, the polyplexes were observed to have small absolute ζ potentials (~

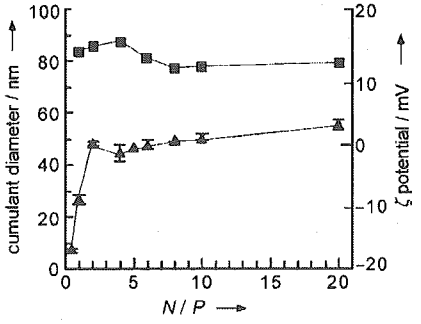


Figure 6. Cumulant diameter (■) and ζ potential (▲) of the PEG-*b*-P[Asp(DET)] polyplex micelles as a function of *N/P* ratio.

8 mV), suggesting a core–shell architecture with a hydrophilic and neutral PEG shell surrounding the polyplex core.

The in vitro transfection efficiency (TE) against human hepatoma HuH-7 cells was assessed by a luciferase assay (Figure 7). Notably, a similar trend in TE was also observed for human

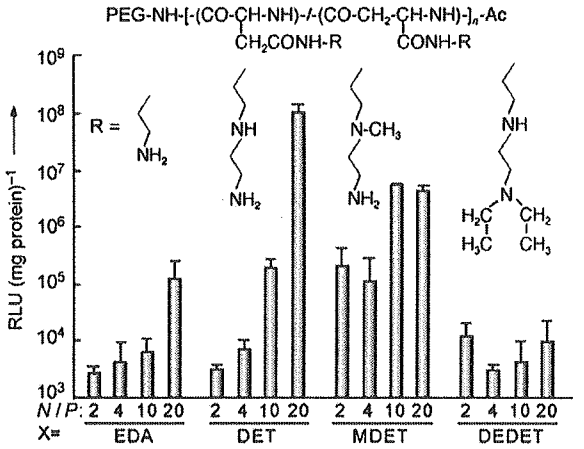


Figure 7. In vitro transfection of the luciferase gene into HuH-7 cells by polyplex micelles from PEG-*b*-polyaspartamides carrying various polyamine components in the side chain (PEG-*b*-P[Asp(X)]) with varying *N/P* ratios. Transfection is reported in relative light units (RLU) per mg protein. The cells were incubated with each polyplex in the medium containing 10% serum for 24 h, followed by incubation for a further 24 h in the absence of polyplex.

kidney 293T cells (Supporting Information). In this experiment, the PEG-*b*-P[Asp(DET)]–pDNA micelle was compared with the polyplex micelles from various PEG-*b*-polyaspartamide catiomers made by the similar aminolysis of PEG-PBLA with different amine compounds, with the aim to highlight the unique nature of the P[Asp(DET)] segment. Note that the polyplex micelles from each block catiomer prepared in this study showed sizes and ζ potentials similar to those of the polyplex micelle from PEG-*b*-P[Asp(DET)] (data not shown). The polyplex micelle from the block catiomer with the 2-aminoethyl group $-(CH_2)_2NH_2$ (pK_a 9.4) in the side chain (PEG-*b*-P[Asp(EDA)]), which was prepared through the aminolysis of PEG-*b*-PBLA with ethylenediamine (EDA), showed only 1/10000 of the TE compared with the PEG-*b*-P[Asp(DET)] polyplex micelle at $N/P = 20$. This is presumably due to the impaired buffering capacity of the $-(CH_2)_2NH_2$ unit with the high pK_a value of 9.4 in the experimental pH range as well as to the weak ability of PEG-*b*-P[Asp(EDA)] to condense pDNA based on the EtBr exclusion assay (data not shown).

The TE of the PEG-*b*-P[Asp(DET)] polyplex micelle was further compared with those of the polyplex micelles from the PEG-*b*-polyaspartamide catiomers carrying the N-alkylated ethylenediamine units in the side chain to explore the structural features of the polyplex micelles that are important for effective gene transfection (Figure 7). These block catiomers, PEG-*b*-P[Asp(MDET)] and PEG-*b*-P[Asp(DEDET)], are prepared by the aminolysis reaction of PEG-PBLA with the corresponding amine compounds, 4-methyldiethylenetriamine (MDET) and *N,N*-diethyldiethylenetriamine (DEDET), respectively. Both the PEG-*b*-P[Asp(MDET)] and PEG-*b*-P[Asp(DEDET)] polyplex micelles showed an appreciably lower TE than the PEG-*b*-P[Asp(DET)] polyplex micelle, particularly at higher *N/P* ratios (Figure 7). This result, which highlights the critical sensitivity of TE toward subtle changes in catiomer structure, indicates that additional structural factors, besides distinct pK_a values, play a substantial role in determining the TE of the polyplex micelles constructed from the PEG-*b*-polyaspartamide catiomers carrying the derivatized ethylenediamine units as a side chain; further study is needed to clarify the detailed mechanisms.

The cytotoxicity of the polyplex-forming catiomers is also a crucial aspect for successful nonviral gene therapy. In this regard, all the polyplex micelles from each block catiomer shown in Figure 7 elicited no appreciable cytotoxicity toward HuH-7 cells under the same conditions used for gene transfection (data not shown). Notably, the polyplex micelle from PEG-*b*-P[Asp(DET)] showed remarkably low cytotoxicity despite its efficiency in gene transfection. Therefore, the intrinsic cytotoxicity of PEG-*b*-P[Asp(DET)] catiomer was further assessed against HuH-7 cells, and compared with that of branched polyethylenimine (BPEI, 25 kDa, Aldrich Chemical, USA) and linear polyethylenimine (LPEI, 22 kDa, ExGen500, MBI Fermentas, Germany). As shown in Figure 8, the PEG-*b*-P[Asp(DET)] catiomer

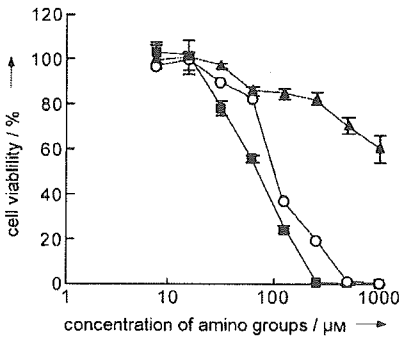


Figure 8. Cytotoxicity of branched (BPEI, ○) and linear (LPEI, ■) polyethylenimines and PEG-*b*-P[Asp(DET)] (▲) against HuH-7 cells. The cells were incubated with each catiomer with different concentrations for 48 h.

showed >20-fold higher 50% growth-inhibitory concentration (IC_{50}) than BPEI and LPEI, highlighting the remarkably low cytotoxicity of the block catiomers synthesized in this study.

The major challenge for the practical use of synthetic vectors in gene therapy is the effective and non-cytotoxic gene transfer to primary cells with therapeutic interest. To evaluate the feasibility of the PEG-*b*-P[Asp(DET)] polyplex micelles toward such primary cells, mouse primary osteoblasts, which are the focus of clinical interest in bone regeneration,[16] were challenged with the polyplex micelles. The luciferase plasmid was transfected, and the resulting TE and cytotoxicity profiles are shown in Figure 9. Notably, the PEG-*b*-P[Asp(DET)] system with $N/P = 80$ gave a TE similar to the polyplexes from ExGen500, the most effective transfection reagent based on LPEI,[17] with the optimal *N/P* ratios (Figure 9 A). Nonetheless,

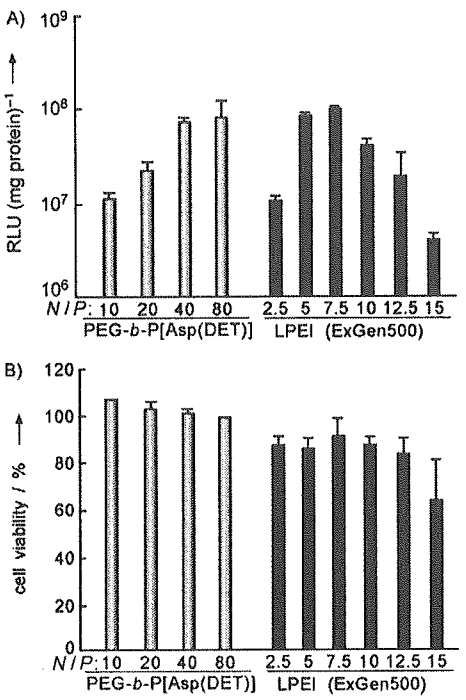


Figure 9. A) In vitro transfection efficiency and B) cytotoxicity of the PEG-*b*-P[Asp(DET)] polyplex micelles and LPEI polyplexes with varying *N/P* ratios toward mouse primary osteoblasts after a 48 h incubation.

the PEG-*b*-P[Asp(DET)] system exhibited no appreciable cytotoxicity under the conditions of gene transfection (Figure 9B). Thus, we have successfully obtained highly transfection-efficient and less toxic polyplex micelles in this study. Particularly, the less toxic nature of the block catiomers compared with conventional catiomers of high transfection efficiency, as observed in Figure 8, should be of great significance for in vivo nonviral gene therapy. Indeed, bone regeneration in critical-size cranial defects based on in vivo transduction of osteogenic factors was recently carried out by our research group by using the PEG-*b*-P[Asp(DET)] polyplex micelle with plasmids expressing the optimized combination of osteogenic factors to facilitate cellular differentiation in situ.[18] Furthermore, polyplex micelles with the PEG palisade seem to be suitable for systemic gene delivery,[7,11] and the engineering the constituent block catiomers to construct polyplex micelles with integrated smart functions such as environment sensitivity[8,10] and tissue targetability[9] will maximize the efficacy of nonviral gene therapy. Thus, the PEG-*b*-P[Asp(DET)] polyplex micelle is expected to be a biocompatible vector system applicable toward various aspects of gene medicine.

Conclusion

We have established a simple and novel synthetic route for the generation of biocompatible block catiomers through the quantitative aminolysis of PEG-*b*-PBLA. The construction of a library of block catiomers, the PEG-*b*-polyaspartamides carrying a series of amine compounds in the side chain, revealed the importance of the ethylenediamine unit for enhanced and less toxic gene transfection by the polyplex micelles made from pDNA and the block catiomers. The availability of the polyplex micelles developed in this study for the transfection of primary osteoblasts will facilitate the use of this type of block catiomer for the construction of synthetic vectors suitable for nonviral gene therapy.

Experimental Section

Materials: β-Benzyl-L-aspartate *N*-carboxyanhydride (BLA-NCA) and α-methoxy-ω-amino poly(ethylene glycol) (MeO-PEG-NH_2) (M_r = 12000) were obtained from Nippon Oil and Fats (Tokyo, Japan). Ethylenediamine (EDA), diethylenetriamine (DET) and 4-methyldiethylenetriamine (MDET) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and distilled over CaH_2 under decreased pressure. *N,N*-Diethyldiethylenetriamine (DEDET) was purchased from Lancaster Synthesis, (Lancashire, England) and distilled over CaH_2 under decreased pressure. *N,N*-Dimethylformamide (DMF), dichloromethane, and acetic anhydride were purchased from Wako Pure Chemical Industries, (Osaka, Japan) and purified by general methods before use.

Synthesis of PEG-*b*-polyaspartamide catiomers: The PEG-*block*-poly(β-benzyl L-aspartate) (PEG-*b*-PBLA) copolymer was prepared as previously reported.[19] Briefly, BLA-NCA was polymerized in DMF at 40 °C by the initiation from the terminal primary amino group of MeO-PEG-NH_2, followed by acetylation of the *N*-terminus of PBLA to obtain PEG-*b*-PBLA. PEG-*b*-PBLA was confirmed to have a unimodal molecular weight distribution (M_w/M_n: 1.17) by gel-permeation chromatography (GPC) measurement (columns: TSK-gel G4000HHR + G3000HHR, eluent: DMF + 10 mM LiCl, T = 40 °C, detector: RI) (data not shown). The degree of polymerization (DP) of PBLA was calculated to be 68 based on ^{1}H NMR spectroscopy (data not shown).

Lyophilized PEG-*b*-PBLA (300 mg, 11.6 μmol) was dissolved in DMF (10 mL), followed by reaction with DET (50 equiv to benzyl group of PBLA segment, 4.0 g, 39.4 mmol) under mild anhydrous conditions at 40 °C to obtain PEG-*b*-P[Asp(DET)]. After 24 h, the reaction mixture was slowly added dropwise into a solution of acetic acid (10% *v/v*, 40 mL) and dialyzed against a solution of 0.01 N HCl and distilled water (M_r cutoff: 3500 Da). The final solution was lyophilized to obtain the polymer as the chloride salt form, and the yield was approximately 90%. Similarly, other block catiomers, PEG-*b*-P[Asp(EDA)], PEG-*b*-P[Asp(MDET)], and PEG-*b*-P[Asp(DEDET)] were synthesized by the aminolysis reaction of PEG-*b*-PBLA with EDA, MDET, and DEDET, respectively. The structures of these block catiomers were confirmed by ^{1}H and ^{13}C NMR measurements and size-exclusion chromatography (SEC).

Potentiometric titration of block catiomers: PEG-*b*-P[Asp(DET)] (30 mg) was dissolved in 50 mL 0.01 N HCl and titrated with 0.01 N NaOH. An automatic titrator (TS-2000, Hiranuma, Kyoto, Japan) was used for titration. In this experiment, the titrant was added in quantities of 0.063 mL after the pH values were stabilized (minimal interval: 30 s). The α/pH curves were determined from the obtained titration curves.

Dye exclusion assay: Polyplex solutions with a pDNA concentration of 33 μg mL^{-1}, prepared by mixing pDNA and block catiomers at different *N/P* ratios (*N* = total amines in block catiomer; *P* = total phosphate anions in pDNA), were diluted to 10 μg pDNA mL^{-1} with ethidium bromide (EtBr, 2.5 mg mL^{-1}) in 10 mM Tris–HCl

