**Review**

Possible Mechanisms of Practical Thresholds for Genotoxicity

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(Received September 24, 2008; Revised October 10, 2008; Accepted October 15, 2008)

An axiom in regulatory sciences is that there are no thresholds for genotoxicity of chemicals. It leads to another default assumption that genotoxic carcinogens impose cancer risk on humans without thresholds, i.e., a linear non-threshold model. Therefore, no acceptable daily intake (ADI) is set for food additives, pesticides and veterinary drugs when they have genotoxic and carcinogenic activities. However, humans possess a number of defense mechanisms such as metabolic inactivation, DNA repair, error-free translesion DNA synthesis, and so on. These mechanisms may constitute practical thresholds for genotoxicity. Error-free translesion DNA synthesis is a process where DNA polymerases bypass lesions in DNA by insertion of correct bases opposite the lesion and continue replication of whole chromosomes. These mechanisms might have been evolved because organisms from bacteria to humans are exposed to endogenous as well as exogenous genotoxic compounds. In fact, levels of spontaneous mutagenesis are strongly influenced by ability of DNA repair and translesion DNA synthesis of the host cells.

Here, I show evidence that DNA repair and translesion DNA synthesis play roles in practical genotoxic thresholds in *Salmonella typhimurium* used for bacterial mutation assays, and discuss future directions of the research on genotoxic thresholds in vivo.

**Key words:** genotoxic thresholds, DNA repair, translesion DNA synthesis, ADI

**Introduction**

Human chromosome is exposed to a variety of endogenous and exogenous agents (1,2). The most prominent endogenous genotoxic agents are reactive oxygen species (ROS), which are generated as by-products of oxygen metabolism (3,4). These reactive molecules include superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen. ROS is also generated in cells by exposure to radiation and chemical carcinogens. Because ROS damages nearby cellular components such as DNA, proteins and lipids in membrane, cells must have evolved multiple defense mechanisms to combat the oxidative stress. Enzymes such as catalase or superoxide dismutase detoxify ROS, and low-molecular-weight scavengers such as glutathione alleviate the toxicity of ROS. Nevertheless, some ROS molecules escape from the defense systems and inevitably damage the biomolecules. DNA repair mechanisms, e.g., 8-hydroxyguanine (8-OH-G) DNA glycosylase encoded by *OGG1* in humans and *mutM* in *Escherichia coli*, remove the damage and convert the modified bases to unmodified ones (5,6).

Another class of endogenous genotoxic agents is alkylating agents such as S-adenosylmethionine (SAM) (7). SAM is an S_n2-(bimolecular) alkylating agent and induces 7-methylguanine and 3-methyladenine in DNA non-enzymatically (8). Although 7-methylguanine is formed more abundantly than 3-methyladenine, it is an innocuous modified base. 3-Methyladenine in DNA blocks DNA replication and is cytotoxic. It is estimated that about 600 3-methyladenine residues are formed by SAM in the DNA of a mammalian cell per day (9). Other S_n2-alkylating agents, e.g., naturally occurring methyl halides, induce N^1^-methyladenine and N^3^-methylcytosine in particular in single-stranded DNA (10). Endogenous S_n1-(monomolecular) alkylating agents such as nitrosamines may induce O^6^-methylguanine and O^4^-methylthymine, which are mutagenic and toxic lesions in DNA. As in the case of ROS, cells possess a number of defense mechanisms against alkylation damages in DNA (10). O^6^-methylguanine DNA methyltransferase (MGMT) directly removes methyl groups from O^6^-methylguanine and O^4^-methylthymine, and 3-methyladenine DNA glycosylase excises 3-methyladenine from DNA, followed by gap-filling by DNA polymerases (DNA Pols). AlkB in *E. coli* and the counterparts in humans, i.e., ABH2 and ABH3, oxidize methyl groups modified in N^1^-adenine and N^2^-cytosine, and remove the methyl groups, thereby reverting them into intact adenine and cytosine bases, respectively.
In addition to endogenous genotoxic agents, human DNA is damaged by exogenous chemical and physical genotoxic insults (11). These include ultraviolet light (UV), radiation, cigarette smoke, polluted air, mutagenic heterocyclic amines, asbestos and so on. The detrimental factors induce bulky DNA adducts, single- or double-strand breaks in DNA. Again, humans possess repair mechanisms against the lesions, such as nucleotide excision repair and homologous or non-homologous recombination, which are responsible for the repair of bulky DNA adducts and strand breaks in DNA, respectively (12). Even when adducts in DNA are not removed, error-free translesion DNA synthesis (TLS) bypasses the damage, thereby reducing the chance of induction of mutations and chromosome aberrations (13) (see below for more detail).

Here, I discuss the possibility that the abovementioned defense mechanisms, i.e., DNA repair and translesion DNA synthesis, may contribute to establish “practical thresholds” for genotoxicity. The term “practical thresholds” is defined as the doses below which no mutations are detectable (14,15). We developed sets of repair-deficient derivatives of \textit{Salmonella typhimurium} TA1535, which is widely used in Ames genotoxicity assay, and used them for demonstration of the practical thresholds for genotoxicity (16–18). I also briefly discuss the \textit{in vivo} (mouse) research on genotoxic thresholds.

**MGMT is a Constituent of Oractical Thresholds for Alkylation-induced Genotoxicity**

First, we constructed an MGMT-deficient derivative of strain TA1535, namely YG7108, and compared the dose-responses against alkylation agents (16,17) (Fig. 1). MGMT is encoded by two genes in \textit{Salmonella}, namely \textit{adaST} and \textit{ogtST} (16,19). Both gene products remove mutagenic lesion, i.e., Oβ-methyl, ethyl, propyl and butyl guanine in DNA, which are induced by a variety of alkylation agents. The alkylation agents used in the study are N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), N-ethyl-N′-nitro-N-nitrosoguanidine (ENNG), N-propyl-N′-nitro-N-nitrosoguanidine (PNNG), N-butyl-N′-nitro-N-nitrosoguanidine (BNNG) and methyl methanesulfonate (MMS). MNNG, ENNG, PNNG and BNNG are different in the length of alkyl chains and MMS induces 3-methyladenine, a cytotoxic lesion, in addition to Oβ-methylguanine. The \textit{ada} and \textit{ogt}-deficient strain YG7108 exhibited superior sensitivity to the genotoxicity of all the alkylation agents used compared to the repair proficient strain TA1535. In particular, the mutagenicity of ENNG and MMS is clear in YG7108 while the mutagenicity is almost completely suppressed in the repair proficient strain TA1535. In the low dose range of MNNG, the mutagenicity was only observed with YG7108 but not TA1535. These results strongly suggest that MGMT is a constituent of practical thresholds for alkylation agents in \textit{Salmonella} strains.

**8-OH-G DNA Glycosylase is a Constituent of Practical Thresholds for Oxidation-induced Genotoxicity**

Next, we compared the dose responses between \textit{mutMST}-deficient and proficient derivatives of \textit{S. typhimurium} TA1535 and TA1975, i.e., YG3001 and YG3002, respectively, against oxidative mutagens (Fig.
2). The mutMST gene encodes 8-OH-G DNA glycosylase in S. typhimurium (18). Both YG3001 and YG3002 are deficient in mutMST but YG3001 is also deficient in functions of nucleotide excision repair (ΔuvrB). The oxidative mutagens used are potassium bromate, methylene blue (MB) plus visible light, benzo[a]pyrene (BP) plus visible light and 4-nitroquinoline N-oxide (4-NQO). Potassium bromate is a rat renal carcinogen and induces 8-OH-G in DNA (20). MB is a photosensitizer and induces 8-OH-G in DNA in the presence of visible light (MB plus visible light) (21). BP is a well known genotoxic carcinogen upon metabolic activation, but the mutagenicity was assayed without metabolic activation in this study (22). Instead, BP was activated by exposure to visible light (BP plus visible light). 4-NQO is a genotoxic carcinogen too. Although 4-NQO induces bulky DNA adducts and oxidative lesions (23), the bulky adducts are removed by nucleotide excision repair in the backgrounds of TA1975 and YG3002 and thus the mutagenicity in the backgrounds depends on the oxidative lesion, namely 8-OH-G in DNA. The mutMST-deficient stains exhibited much higher sensitivity compared to the proficient strains (18). The mutagenicity of potassium bromate and 4-NQO was clearly observed in the mutMST-deficient stains, i.e., YG3001 and YG3002, respectively, while the mutagenicity was almost completely suppressed in the proficient strains, i.e., TA1535 and TA1975. Strain YG3001 also exhibited much higher sensitivity against MB plus visible light and BP plus visible light. These results suggest that 8-OH-G DNA glycosylase contributes to establish practical thresholds against oxidative mutagens.

8-OH-G DNA glycosylase is present not only in bacteria but also in humans (24). The glycosylase in humans is encoded by OGG1. Interestingly, there is a genetic polymorphism in the human OGG1 gene (25). We conducted a functional complementation assay where three polymorphic forms of human OGG1, i.e., hOGG1-Ser326, hOGG1-Cys326 and hOGG1-Gln46, are expressed in Salmonella strain YG3001 deficient in the bacterial mutMST gene and the mutagenicity of MB plus visible light was assayed with the strains (26). Although human OGG1 proteins suppressed the photomutagenicity of MB, the extent of suppression was different among three polymorphic forms where hOGG1-Gln46 exhibited the weakest suppression (hOGG1-Ser326 and hOGG1-Cys326 have Arg at amino acid 46 and hOGG1-Gln46 has Ser at amino acid 326). The results suggest that each polymorphic form of OGG1 may have different ability to suppress mutations induced by the oxidative DNA damage and also that the genetic polymorphism may affect the practical thresholds for oxidative mutagenesis.

TLS may be a Constituent of Practical Thresholds for Genotoxicity

Recent progress in research on DNA Pols revealed that humans possess more than 14 DNA Pols per cell and about half of them participate in DNA repair and TLS (27). TLS is a process where DNA Pols continues DNA synthesis across lesions (11). If correct bases are inserted opposite the lesions, TLS will reduce the chance of induction of mutations and contribute to DNA damage tolerance. However, if incorrect bases are in-
serted opposite the lesions or skip the lesion, it will induce point mutations such as base substitutions or frameshifts. If no TLS occurs, DNA replication may stall and DNA strands may be broken, which leads to chromosome aberrations. Therefore, TLS is a critical molecular event whether DNA damage is converted to mutations including chromosome aberrations or not. Even in *S. typhimurium*, whose genome size is about 1/1,000 of the size of human genome, there are six DNA Pols (13,28). Five of the Pols are encoded by the genes in the chromosome and the remaining one is encoded by the gene on the cryptic plasmid (29). Moreover, there is an additional plasmid pKM101 in *S. typhimurium* TA98 and TA100, where the *mucAB* genes encoding DNA Pol R1 are present (30). The presence of plasmid pKM101 carrying the *mucAB* genes strongly affects the sensitivity of *S. typhimurium* strains to a variety of chemical. In particular, the mutagenicity of furylfuramide (AF-2) and aflatoxin B1 can be clearly detected with strain TA100 harboring plasmid pKM101 while no mutagenicity is observed with strain TA1535, the same as TA100 but has no plasmid pKM101 (31). AF-2 is a food additive that has been banned in Japan because of the carcinogenicity in the mice, and aflatoxin B1 is a fungal toxin that can induce liver tumors in humans. It is supposed that DNA Pol R1 bypasses DNA adducts induced by AF2 and aflatoxin B1 in an error-prone manner while other six DNA Pols in *S. typhimurium* can not. In contrast, human DNA Pol η is responsible for protection of genomic DNA from mutagenic effects of UV (32,33). This enzyme carries out error-free TLS across pyrimidine dimers in DNA and reduces the chance of mutations induced by UV. Lack of DNA Pol η leads to induction of Xeroderma pigmentosum variant, which is a genetic disease whose patients are highly sensitive to sunlight-induced skin cancer. Interestingly, bacterial DNA Pol R1 and human DNA Pol η belong to the same family of DNA Pol, i.e., Y-family. Therefore, TLS mediated by Y-family DNA Pols may enhance or reduce the frequencies of mutations, thereby influencing the practical thresholds for genotoxicity.

**Both DNA Repair and Error-prone TLS Affect Levels of Spontaneous Mutagenesis**

As written in Introduction, chromosome DNA is continuously exposed to not only exogenous genotoxic agents but also to endogenous ones. These endogenous lesions are causes for so-called spontaneous mutations (1). Interestingly, both DNA repair and TLS play important roles in regulations of spontaneous mutagenesis. When *adfs* and *ogfs* encoding MGMT are deleted in *S. typhimurium* TA1535, the number of spontaneous His+ revertants per plate increases two- to three-fold (16). Similar extent of an increase in the number of spontaneous revertants per plate was observed in ΔmutMST strain, i.e., YG3001 (18). Introduction of plasmid pKM101 enhances the number of spontaneous revertants per plate of strain TA1535 more than five times (31). Both deletions of the repair genes and introduction of plasmid pKM101 exhibit additive effects on the spontaneous mutagenesis. These results suggest that the levels of spontaneous mutagenesis, which may play important roles in determination of threshold levels for genotoxicity, is strongly affected by the ability to repair DNA damage and to bypass lesions by DNA Pols in host cells.

**Discussion**

In theory, even a single molecule of mutagens could interact with DNA and induce genetic alterations, which might lead to cancer (15). Therefore, it is supposed that there are no thresholds for the risk of genotoxic and carcinogenic compounds and also that even a small amount of such compounds can impose carcinogenic loads on humans. Because of the assumption, no accepted daily intake (ADI) is set for food additives, pesticides and veterinary drugs when they have genotoxic and carcinogenic activities. The assumption is counterintuitive, however, because humans possess a number of defense mechanisms against endogenous and exogenous genotoxic insults. The mechanisms include antioxidants, detoxification metabolisms, DNA repair and error-free TLS (Fig. 3). These mechanisms may suppress genotoxicity and reduce it below the detection limits. In fact, both MGMT and 8-OH-G DNA glycosylase strongly affect the sensitivity of *S. typhimurium* strains for genotoxicity assays, thereby suggesting the possibility that they may be constituents of practical thresholds for genotoxicity. In some cases, however, that linear non-

![Genotoxicity](#)

Fig. 3. Possible mechanisms underlying practical thresholds for genotoxicity. Detoxication mechanisms inactive genotoxic compounds. When active genotoxic compounds induce DNA adducts, DNA repair mechanisms remove them, thereby reverting the modified bases into intact ones. Error-free translation DNA synthesis (TLS) inserts correct bases opposite the lesions and reduces the chance of induction of mutations. Therefore, these mechanisms, i.e., metabolic inactivation, DNA repair and error-free TLS, may be constituents of practical thresholds for genotoxicity. In contrast, error-prone TLS enhances mutations by insertion of incorrect bases opposite the lesions or skipping the lesions. These molecular events lead to base substitutions and frameshift mutations, respectively.
threshold dose response can be observed for genotoxicity even in the presence of wild-type DNA repair (34). Although error-free TLS can reduce the levels of mutations, error-prone TLS has an opposite effect and enhances the sensitivity to genotoxic compounds. Genetic approaches with cells deficient in DNA repair capacity and/or TLS are powerful tools to analyze possible mechanisms underlying practical thresholds for genotoxicity. Since risk assessment of chemical carcinogens is usually conducted with experimental animals, i.e., rats and mice, it is necessary to expand the genetic approaches to in vivo. In this respect, gpt delta rats and mice may be useful backgrounds to investigate constituents of the practical thresholds (35,36). These transgenic rodents harbor reporter genes for mutations, which can enable to identify genotoxicity in target organs of chemical carcinogens (37). So far, gpt delta mice have been crossed with a number of knockout mice such as p53, Ogg1, Parp-1, Atm, IL-10 and Nrf-2 (38–43). It is important to examine which factors, e.g., detoxication, DNA repair or TLS, most strongly affect the levels of practical thresholds for genotoxicity and carcinogenicity in vivo. These studies are currently in progress in our laboratory.

Acknowledgements: Part of this work is financially supported by grant-in-aid by the Ministry of Health, Labour and Welfare, Japan.

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