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# Repair of $O^6$-ethylguanine in DNA protects rat 208F cells from tumorigenic conversion by *N*-ethyl-*N*-nitrosourea

($O^6$-alkylguanine-DNA alkyltransferase/repair-deficient and -proficient variants/anti-alkyldeoxynucleoside monoclonal antibodies)

JÜRGEN THOMALE, NAM-HO HUH*, PETER NEHLS†, GERTRUD EBERLE, AND MANFRED F. RAJEWSKY‡

Institute of Cell Biology (Cancer Research), University of Essen Medical School, Hufelandstrasse 55, D-4300 Essen 1, Federal Republic of Germany

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ABSTRACT **$O^6$-Ethylguanine ($O^6$-EtGua) is one of about a dozen different alkylation products formed in the DNA of cells exposed to the alkylating *N*-nitroso carcinogen *N*-ethyl-*N*-nitrosourea (EtNU). We have evaluated selectively the relative capacity of cells for the specific enzymatic repair of $O^6$-EtGua as a determinant for the probability of malignant conversion. Eleven $O^6$-EtGua-repair-proficient ($R^+$) variant subclones were isolated from the $O^6$-EtGua-repair-deficient ($R^-$) clonal rat fibroblast line 208F by selection for resistance to 1,3-bis-(2-chloroethyl)-1-nitrosourea (frequency, $\approx 10^{-5}$). Contrary to the 208F wild-type cells, all variants expressed $O^6$-methylguanine-DNA methyltransferase activity, while both kinds of cells were deficient for repair of the DNA ethylation products $O^2$- and $O^4$-ethylthymine. After exposure to EtNU ($\leq$500 $\mu$g/ml; 20 min), cells were analyzed for the formation of piled-up foci in monolayer culture and of anchorage-independent colonies in semisolid agar medium. Depending on the EtNU concentration, the frequencies of piled-up foci and agar colonies, respectively, in the $R^+$ variants were as low as 1/28th and 1/56th of those in the $R^-$ wild type. Contrasting with the cells from $R^+$ variant-derived agar colonies, cells from 208F ($R^-$) agar colonies gave rise to highly malignant tumors when implanted subcutaneously into syngeneic rats. No significant differences in the frequencies of piled-up foci were found between wild-type and variant cells after exposure to the major reactive metabolite of benzo[*a*]pyrene, (+)-7$\beta$,8$\alpha$-dihydroxy-9,10$\alpha$-epoxy-7,8,9,10$\alpha$-tetrahydrobenzo[*a*]pyrene, for which stable binding to guanine $O^6$ in cellular DNA has not been observed. The relative capacity of cells for repair of $O^6$-alkylguanine is, therefore, a critical determinant for their risk of malignant conversion by *N*-nitroso carcinogens.**

In replication-competent cells exposed to DNA-reactive carcinogens the relative capacity for error-free enzymatic repair of potentially mutagenic DNA lesions may be a principal determinant of transformation risk. This assumption is borne out, albeit indirectly, by the predominant occurrence of malignant tumors in the brain and peripheral nervous system of rats after a single pulse of the *N*-nitroso carcinogen *N*-ethyl-*N*-nitrosourea (EtNU) applied systemically during prenatal or early postnatal development (1, 2). In this system, the cell type-selective carcinogenic effect is correlated with the persistence of the miscoding alkylation product $O^6$-ethylguanine ($O^6$-EtGua) in brain DNA; contrary to the cells of other rat tissues, rat brain cells are $O^6$-EtGua-repair-deficient ($R^-$) (3).

$O^6$-EtGua is one of about a dozen ethylation products formed in DNA upon exposure to EtNU (3, 4), all of which have been well characterized and many of which can be quantitated with high specificity and sensitivity by using anti-alkyldeoxynucleoside monoclonal antibodies (mAbs) (5). As in bacterial DNA (6, 7), $O^6$-alkylguanine ($O^6$-AlkGua) in the DNA of mammalian cells is efficiently repaired by an $O^6$-alkylguanine-DNA alkyltransferase (AGT; EC 2.1.1.63) (7–10). The AGT restores the integrity of the guanine base in a single step by stoichiometrically transferring one alkyl group per AGT molecule from the $O^6$ atom of guanine to one of its cysteine residues, thereby becoming inactivated (suicide enzyme). While AGTs from *Escherichia coli* and *Bacillus subtilis* have been characterized and sequenced (11–13), cDNA clones encoding AGTs from mammalian cells have only recently been obtained (14–16) and the regulation of the respective gene(s) remain(s) to be clarified.

AGT activity varies considerably among mammalian species and also interindividually, between different types of cells, and between malignant tumors (2, 3, 7, 9, 17–21), implying differential susceptibility to alkylating agents in terms of mutagenicity, neoplastic transformation, and cytotoxicity [e.g., of *N*-nitroso compounds used for cancer therapy (22)]. We have attempted to evaluate directly and specifically the relative importance of repair versus persistence of $O^6$-AlkGua in genomic DNA for the transformation risk of EtNU-exposed cells. A panel of individual $O^6$-EtGua repair-proficient ($R^+$) variant subclones were isolated from the clonal rat fibroblast cell line 208F ($R^-$), via selection for resistance to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). Following exposure to EtNU, comparative analyses were performed in $R^+$ variants and $R^-$ wild-type (WT) cells using several indicators of transformation [i.e., the frequencies of both piled-up foci in monolayer cultures and anchorage-independent colonies (AI-clones) in semisolid agar medium, and tumorigenicity in syngeneic animals after reimplantation of cells derived from AI-clones]. All of these indicators showed considerably lower values for the $R^+$ variants, demonstrating that the relative capacity of cells for enzymatic repair of $O^6$-EtGua in DNA is indeed a critical determinant for the probability of transformation by *N*-nitroso carcinogens.

## MATERIALS AND METHODS

**Cells and Cell Culture.** The nontumorigenic cell line 208F, a subclone of the Fischer rat embryo fibroblast line F2408

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Abbreviations: AGT, $O^6$-alkylguanine-DNA alkyltransferase; AI-clones, anchorage-independent clones; BPDE-I, (+)-7$\beta$,8$\alpha$-dihydroxy-9,10$\alpha$-epoxy-7,8,9,10$\alpha$-tetrahydrobenzo[*a*]pyrene; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; EtNU, *N*-ethyl-*N*-nitrosourea; FCS, fetal calf serum; mAb, monoclonal antibody; $O^6$-AlkGua, $O^6$-alkylguanine; $O^6$-EtdGuo, $O^6$-ethyl-2′-deoxyguanosine; $O^2$-EtdThd, $O^2$-ethylthymidine; $O^4$-EtdThd, $O^4$-ethylthymidine; $O^6$-EtGua, $O^6$-ethylguanine; $O^2$-EtThy, $O^2$-ethylthymine; $O^4$-EtThy, $O^4$-ethylthymine; $R^-$, $O^6$-EtGua-repair-deficient; $R^+$, $O^6$-EtGua-repair-proficient; WT, wild-type.

*Present address: Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan.
†Present address: Abteilung für Molekularbiologie und Allgemeine Pathologie, Universität Ulm, Neuherbergstrasse 11, D-8000 München 45, Federal Republic of Germany.
‡To whom reprint requests should be addressed.

(23), was obtained from R. Müller (University of Marburg, Marburg, F.R.G.). 208F monolayer cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS; Biochrom, Berlin, F.R.G.), penicillin (100 units/ml), and streptomycin (50 μg/ml).

**Isolation of BCNU-Resistant Clones.** 208F cells were seeded at $10^5$ cells per 90-mm dish. After 24 hr of culture, the medium was replaced by FCS-free DMEM containing different concentrations of freshly diluted BCNU (Bristol-Meyers), and the cells were incubated in this medium for 1 hr. Thereafter, the medium was exchanged for BCNU-free serum-containing DMEM. BCNU-resistant colonies were isolated after a further incubation for 2 weeks (24).

**Repair Kinetics of the DNA Ethylation Products $O^6$-EtGua, and $O^2$- and $O^4$-Ethylthymine ($O^2$-EtThy and $O^4$-EtThy).** Cells were exposed to EtNU (Roth, Karlsruhe, F.R.G.; recrystallized twice from methanol; 100 μg/ml) for 20 min (25). High molecular weight DNA was isolated by a standard phenol procedure at different times after the EtNU pulse. The purity and concentrations of DNA were determined by HPLC analysis of enzymatically hydrolyzed aliquots (25, 26). The respective ethyl-2'-deoxynucleoside/2'-deoxynucleoside molar ratios in the DNA samples were determined by immuno-slot-blot (27), using mAbs specific for $O^6$-ethyl-2'-deoxyguanosine ($O^6$-EtdGuo; mAb ER-6; ref. 5), $O^2$-ethylthymidine ($O^2$-EtdThd; mAb EM-4-1; ref. 28), or $O^4$-ethylthymidine ($O^4$-EtdThd; mAb ER-01; ref. 5). In some experiments $O^6$-EtdGuo was quantitated in parallel by competitive RIA (26).

**Cellular Enzymatic Activity for Repair of $O^6$-EtGua.** An mAb-based immunoassay was used to determine the cellular enzymatic activity for repair of $O^6$-EtGua (29). Cell extracts from semiconfluent monolayer cultures were prepared by sonication (three times for 5 sec each) at 0°C in 50 mM Tris·HCl/500 mM NaCl/1 mM dithiothreitol/1 mM EDTA/5% (vol/vol) glycerol, pH 7.8. Protein concentrations of the extracts were determined by a photometric microassay (Bio-Rad), and DNA concentrations were measured fluorimetrically by using the dye Hoechst 33258 (Calbiochem–Behring) (30). Extracts with protein concentrations between 0.5 and 20 μg were incubated with $^{32}$P-labeled double-stranded DNA fragments containing, on average, one $O^6$-EtGua residue per DNA molecule. DNA fragments were then incubated with mAb ER-6 and subsequently passed through nitrocellulose filters (BA85; Schleicher & Schuell). $O^6$-EtGua-containing DNA fragments complexed to mAb ER-6 are retained on the filters and were quantitated by liquid scintillation spectrometry. The sensitivity of this assay is in the subfemtomole range.

**Gel Electrophoresis of Cellular Proteins Containing $^3$H-Labeled AGT.** *Micrococcus lysodeicticus* DNA (Sigma) was methylated by using *N*-[$^3$H]methyl-*N*-nitrosourea (2.8 Ci/mmol; New England Nuclear; 1 Ci = 37 GBq) (31). After partial depurination (100°C; 2 hr) in TE buffer (10 mM Tris·HCl/1 mM EDTA, pH 7.2), the methylated DNA was washed extensively with TE buffer (Centricon-10 microconcentrator; Amicon). Of the remaining $^3$H radioactivity 45% was in $O^6$-methyl-2'-deoxyguanosine (67 cpm/μg of DNA). Samples of methylated DNA (15 μg; $10^3$ cpm) were incubated with cell extracts for 30 min. Proteins were separated by SDS/PAGE and the $^3$H radioactivity in 2-mm gel slices dissolved in Protosol (New England Nuclear) was determined by liquid scintillation spectrometry. Molecular weight protein standards run on the same gels were visualized by Coomassie blue staining.

**Cytotoxicity of EtNU, BCNU, and (+)-7β,8α-Dihydroxy-9,10α-Epoxy-7,8,9,10α-Tetrahydrobenzo[*a*]pyrene (BPDE-I).** Different numbers of logarithmic-phase 208F cells ($10^2$, 3 × $10^2$, or $10^3$ cells per 60-mm dish) were incubated in DMEM supplemented with 10% FCS for 24 hr, prior to exposure to BCNU or BPDE-I in FCS-free DMEM (60 min), or to EtNU (20 min) in phosphate-buffered saline supplemented with $Ca^{2+}$, $Mg^{2+}$, and 25 mM Hepes, pH 7.2 (25). BPDE-I (a gift from B. Brylawsky, University of North Carolina, Chapel Hill, NC) was diluted in tetrahydrofuran/dimethyl sulfoxide (32), and cells were exposed in the dark. For colony counts, cells were fixed with methanol 7 days after drug treatment and stained with Giemsa stain.

**Piled-Up Foci in Monolayer Cultures and AI-Clones in Semisolid Agar Medium.** Twenty-four hours after seeding of logarithmic-phase cells into Falcon dishes (5 × $10^5$ cells per 100-mm dish), cultures were exposed to different concentrations of EtNU or BPDE-I, respectively, as described above. After 6 weeks, piled-up foci were counted by microscopy. To determine the frequencies of AI-clones formed in semisolid agar medium, cells from day 3 monolayer cultures were suspended in 48-ml flasks (Nunc; $10^5$ and $10^6$ cells per flask) containing DMEM supplemented with 10% FCS, 10 mM Hepes at pH 7.2, and Noble agar (Difco) at a final concentration of 0.15%. AI-clones (diameter > 0.5 mm) were counted after 9 weeks of incubation, transferred to agar-free DMEM by Pasteur pipetting, and propagated for subsequent tumorigenicity testing.

**Tumorigenicity Tests.** The tumor-forming ability of cells derived from AI-clones originating from WT and variant cells exposed to EtNU (100 or 500 μg/ml) was determined by s.c. implantation of $10^5$ and $10^6$ cells from subconfluent monolayer cultures (two implants per animal; two animals per AI-clone) into 9-week-old syngeneic Fischer rats (Zentralinstitut für Versuchstierkunde, Hannover, F.R.G.). Tumor growth was monitored by palpation twice weekly for 25 weeks. Tumors were removed for histological inspection (courtesy of L. D. Leder, Institute of Pathology, University of Essen) when they had reached diameters of 20–30 mm.

## RESULTS

**Repair Characteristics of 208F WT and Variant Cells.** 208F WT cells were found to be almost entirely $R^-$ when the kinetics of enzymatic elimination of $O^6$-EtGua from DNA were measured over a period of 24 hr after a 20-min exposure to EtNU (100 μg/ml) *in vitro* (Fig. 1*A*). Similarly, the potentially miscoding ethylpyrimidines $O^2$-EtThy and $O^4$-EtThy persisted in 208F WT DNA over this period (Fig. 1*B*). Thirteen individual variant subclones ($V_1$–$V_{13}$; frequency, ≈$10^{-5}$) were isolated from 208F WT cells by direct selection for resistance to BCNU (0.2–1.0 mM) (24). Contrasting with the parental 208F WT cells, 11 variant subclones exhibited an $R^+$ phenotype (Fig. 1*A*). After exposure to EtNU, between 86% ($V_2$) and 95% ($V_9$) of the "input" amount of $O^6$-EtGua ($O^6$-EtdGuo/dGuo molar ratio in DNA = 8 × $10^6$, corresponding to 2 × $10^4$ $O^6$-EtdGuo residues per diploid genome) were repaired within 24 hr. As exemplified by the variant subclone $V_9$, $O^2$-EtThy and $O^4$-EtThy persisted unrepaired in both variant and WT DNA (Fig. 1*B*).

The differential $O^6$-EtGua repair characteristics of the 208F WT cells and the $R^+$ variants (Fig. 1*A*) were also reflected by the capacity of protein extracts from these cells to repair $O^6$-EtGua in DNA *in vitro*. As shown in Table 1, the repair activities exhibited by extracts from $R^+$ variants were 5–15 times higher than those of extracts from $R^-$ WT cells. When double-stranded DNA containing $O^6$-[$^3$H]methylguanine was incubated with protein extracts from 208F WT or $V_9$ variant cells and proteins were subsequently separated by SDS/PAGE, the $^3$H radioactivity was associated with a 20- to 25-kDa protein band in the case of the $V_9$ but not the 208F WT extract (Fig. 2). This association of the $^3$H-labeled methyl group from $O^6$-methylguanine with a protein band presumed to contain the AGT (7–10) was not changed by preincubation with DNase I, thus excluding an artifact due to small $^3$H-labeled DNA fragments or DNA–protein complexes. On the

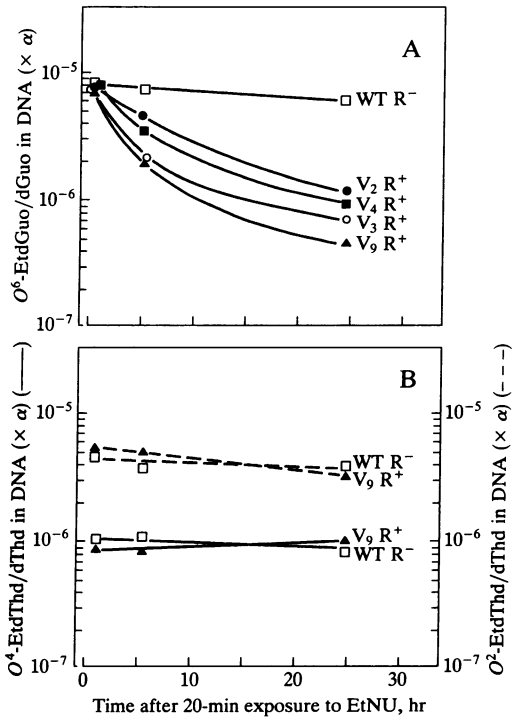


FIG. 1. Kinetics of the elimination of $O^6$-EtGua (*A*) and $O^2$-EtThy (– – – –) and $O^4$-EtThy (———) (*B*) from genomic DNA of 208F WT cells ($R^-$; □) and of the BCNU-resistant 208F variant subclones ($R^+$) $V_2$ (●), $V_3$ (○), $V_4$ (■), and $V_9$ (▲), following a 20-min exposure to EtNU (100 μg/ml) *in vitro*. Ordinates: $O^6$-EtdGuo/dGuo (*A*) and $O^4$-EtdThd/dThd and $O^2$-EtdThd/dThd (*B*) molar ratios in DNA, multiplied by a correction factor α for cell division (25). The values are means of two or more separate experiments.

basis of this analysis, the number of AGT molecules per $V_9$ cell was estimated to be $>2 \times 10^4$.

**Cytotoxicity of BCNU and EtNU in 208F WT Cells and $R^+$ Variants.** When the cytotoxic effects of BCNU and EtNU in 208F WT cells and $R^+$ variants were measured in terms of the reduction of colony-forming capacity as a function of drug concentration, a striking difference between the two agents became apparent. As shown in Fig. 3 for 208F WT, $V_3$, and $V_9$ cells, the cytotoxic effect of BCNU is inversely correlated with the capacity of cells for repair of $O^6$-EtGua. The absence of this correlation after exposure to EtNU indicates that the persistence in DNA of unrepaired $O^6$-EtGua is not *per se* a cause of significant cytotoxicity (33), in contrast to the situation with BCNU, where chloroethylation of the $O^6$ atom of guanine initiates the formation of cytotoxic DNA cross-links (22, 34).

**Formation of Piled-Up Foci and AI-Clones in Semisolid Agar Medium.** The frequencies of piled-up foci and AI-clones were determined as indicators of transformation after exposure of

Table 1. *In vitro* repair of $O^6$-EtGua in double-stranded DNA by extracts from 208F WT cells ($R^-$) and BCNU-resistant 208F variant subclones ($R^+$)

| Cell line | Relative $O^6$-EtGua repair capacity of extract | |
|---|---|---|
| | Based on protein conc. | Based on DNA conc. |
| 208F WT | 1.0 | 1.0 |
| 208F $V_1$ | 4.0 | 4.8 |
| 208F $V_2$ | 4.6 | 4.2 |
| 208F $V_3$ | 8.4 | 7.2 |
| 208F $V_4$ | 6.2 | 5.6 |
| 208F $V_9$ | 13.6 | 15.0 |

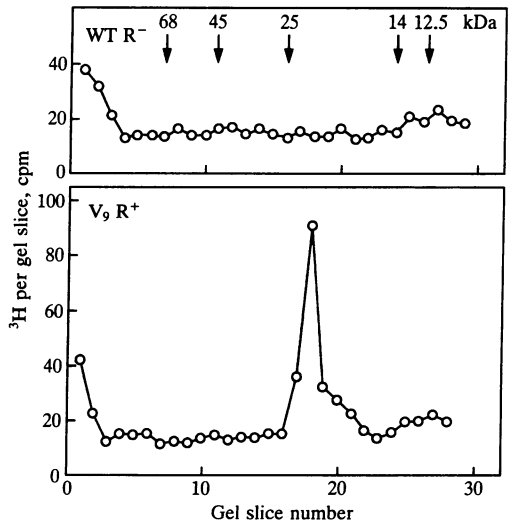


FIG. 2. Analysis of AGT activity in extracts from 208F WT cells ($R^-$) (*Upper*) and from the 208F $V_9$ variant subclone ($R^+$) (*Lower*). Proteins were separated by SDS/PAGE and $^3H$ radioactivity was quantitated in 2-mm gel slices. Arrows indicate locations of marker proteins.

208F WT cells and $R^+$ variants to EtNU at concentrations ranging from 50 to 500 μg/ml. The latter EtNU concentration reduced the survival of colony formation-competent cells to 20% (Fig. 3*A*). Control values (no EtNU treatment) for 208F WT cells or $R^+$ variants were 8 ± 3, (mean ± SD) piled-up foci and 0.7 ± 1.2 AI-clones or 4.3 ± 3.7 piled-up foci and 0.7 ± 0.8 AI-clones, respectively, per $10^6$ cells seeded.

As shown in Fig. 4, both piled-up foci and AI-clones were induced in 208F WT cells at frequencies depending on EtNU concentration, with the frequency of piled-up foci exceeding that of AI-clones (about 5-fold at an EtNU concentration of 500 μg/ml). In contrast, the $R^+$ variants ($V_2$, $V_3$, $V_4$, $V_6$, and $V_9$) exhibited strongly reduced frequencies of piled-up foci and AI-clones, with a poorly resolved dependency on EtNU

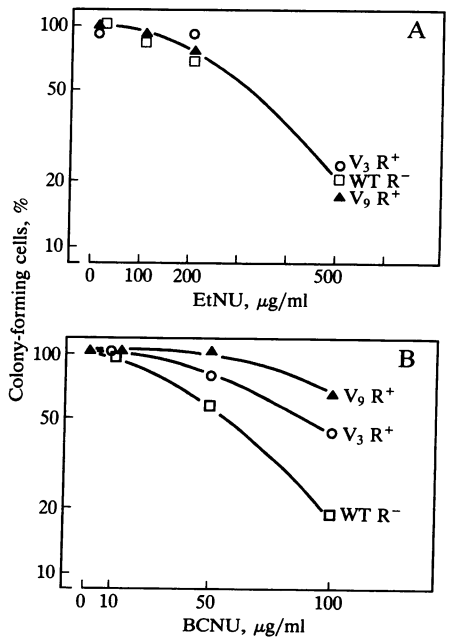


FIG. 3. Cytotoxicity of BCNU and EtNU in $R^-$ 208F (WT) cells (□) and in the $R^+$ 208F variant subclones $V_3$ (○) and $V_9$ (▲). The colony-forming capacity of cells (as determined at 7 days after drug exposure) is expressed in percent of the colony-forming fractions of the respective untreated control cells (51–64%).

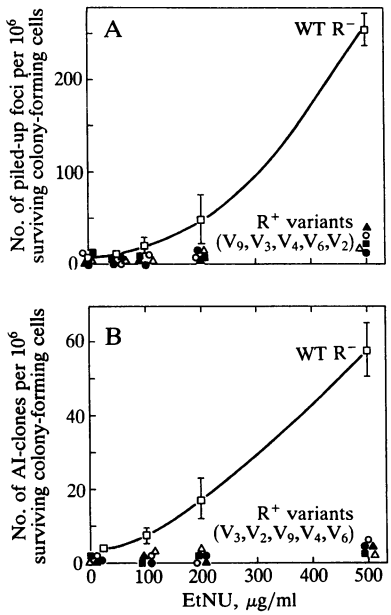


FIG. 4. Formation of piled-up foci (*A*) and AI-clones in semisolid agar medium (*B*) by $R^-$ 208F WT cells (□) and the $R^+$ 208F variants $V_2$ (●), $V_3$ (○), $V_4$ (■), $V_6$ (△), and $V_9$ (▲), after a 20-min exposure to EtNU (50–500 μg/ml). The values are means of triplicate cultures (vertical bars = SD).

concentration. At the highest EtNU concentration, the frequencies of piled-up foci (mean ± SD = 22 ± 12.5 per $10^6$ surviving cells) and AI-clones (5 ± 6 per $10^6$ cells), respectively, were 11.3-fold (range, 6- to 28-fold) and 11.2-fold (range, 7- to 56-fold) lower in the $R^+$ variants in comparison to the corresponding average values for 208F WT cells (248 ± 19 and 56 ± 7, respectively; Fig. 4). Similarly reduced frequencies of piled-up foci and AI-clones were obtained when five other $R^+$ variants were exposed to EtNU at 500 μg/ml (data not shown). The small absolute numbers of piled-up foci and AI-clones in the $R^+$ variants precluded a statistically meaningful correlation with their relative $O^6$-EtGua repair capacity.

To further substantiate the conclusion that the reduced transformation risk of the $R^+$ variants specifically resulted from their capacity for repair of $O^6$-EtGua, the same transformation parameters were compared in 208F WT cells and in the $R^+$ variants $V_3$ and $V_9$ after exposure to the major DNA-reactive metabolite of benzo[*a*]pyrene, BPDE-I, for which stable binding to the guanine $O^6$ atom in cellular DNA has not been reported (35). Contrasting with the results after EtNU treatment (Fig. 4), both types of cells exhibited similar frequencies of piled-up foci as a function of BPDE-I concentration (0–200 ng/ml) (Fig. 5). No AI-clones were formed.

**Tumorigenicity of Cells Derived from 208F WT and Variant ($R^+$) AI-Clones.** Cells derived from both 208F WT and variant AI-clones obtained after exposure to EtNU were tested for tumorigenicity after s.c. implantation of $10^6$ cells into syngeneic Fischer rats. While being equally capable of anchorage-independent proliferation in semisolid agar medium, AI-clones derived from $R^-$ and $R^+$ cells showed a strikingly different behavior in terms of tumorigenicity (Fig. 6). Four out of six AI-clones derived from WT cells produced rapidly growing sarcomas after 4–5 weeks, whereas only two out of eight AI-clones derived from $R^+$ variants were tumorigenic, with slow-growing fibrosarcomas appearing after a much longer latency period (≥13 weeks).

## DISCUSSION

Previous *in vivo* and cell culture experiments have strongly suggested the persistence of unrepaired $O^6$-AlkGua in genomic DNA to be an important determinant of transformation probability in replication-competent target cells exposed to carcinogenic *N*-nitroso compounds (2, 3, 36–41). The presence of $O^6$-AlkGua-derived point mutations (predominantly G·C → A·T transitions) in genes critically associated with carcinogenesis (42) and the crucial role of $O^6$-AlkGua repair—mainly by AGT (7–16)—in the protection of mammalian cells from mutation induction (43) have been elegantly documented. On the other hand, the relative risk of malignant conversion by *N*-nitroso carcinogens had thus far not been compared in phenotypically identical target cells differing only in their capacity for the specific repair of $O^6$-AlkGua.

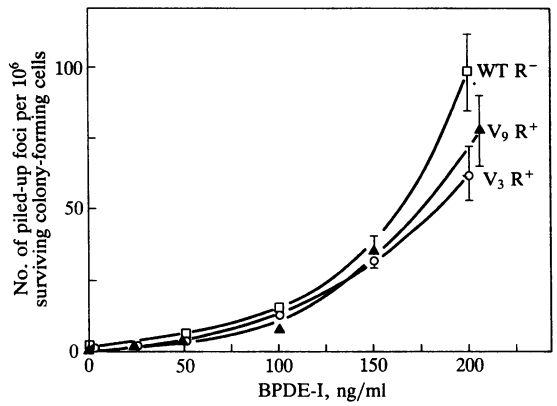


FIG. 5. Formation of piled-up foci by $R^-$ 208F WT cells and the $R^+$ 208F variants $V_9$ and $V_3$, after a 60-min exposure to activated benzo[*a*]pyrene (BPDE-I) (25–200 ng/ml). The values are means of triplicate cultures (vertical bars = SD).

The present approach was prompted by our recent observations (24, 25, 44) and those reported by other groups (45–47), indicating that, at least in transformed cell lines, the expression of AGT is not always a stable phenotypic property. This suggested that—based on relative resistance to BCNU—the isolation of $R^+$ variants occurring at low frequency in an $R^-$ clone of nontumorigenic WT cells (208F) might be possible. The successful isolation of 13 individual $R^+$ variant subclones expressing AGT provided a strong rationale for evaluating in WT $R^-$ cells versus their $R^+$ subclones the capacity for repair of $O^6$-EtGua as a critical variable affecting the transformation parameters chosen. Moreover, there was no difference between variant and WT cells with respect to the relative stability of two other potentially mutagenic ethylation products, $O^4$-EtThy and $O^2$-EtThy, which both persisted in DNA unrepaired. Last, variant and WT cells showed similar frequencies of piled-up

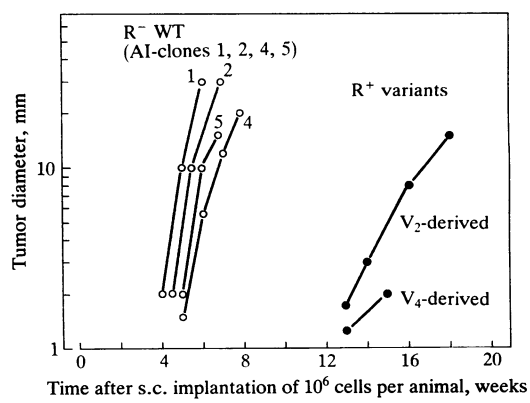


FIG. 6. Kinetics of tumor growth in syngeneic Fischer rats after s.c. implantation of $10^6$ cells derived from AI-clones of EtNU (500 μg/ml)-treated (*i*) $R^-$ 208F WT cells (AI-clones 1, 2, 4, and 5; ○) and (*ii*) $R^+$ 208F variants $V_2$ and $V_4$ (●).

foci (and background levels of AI-clones) upon exposure to a carcinogen (BPDE-I) which does not bind to the $O^6$ atom of guanine in cellular DNA (35).

After exposure to the highest concentration of EtNU (500 $\mu$g/ml), the average frequencies of piled-up foci and AI-clones, respectively, were 11.3- and 11.2-fold higher in WT cells compared with the $R^+$ variants, indicating a strong protective effect of $O^6$-EtGua repair. Nonetheless, the variants still exhibited average frequencies of piled-up foci and AI-clones ($4.3 \times 10^{-6}$ and $0.7 \times 10^{-6}$, respectively) exceeding the corresponding values for untreated $R^+$ cells by factors of 5.1 and 7.1. This implies that mutational events caused by persistent DNA lesions other than unrepaired $O^6$-EtGua residues—e.g., by the unrepaired alkylation products $O^4$-EtThy (48) and possibly $O^2$-EtThy—may also contribute to the risk of malignant conversion. However, in view of the $\approx$2.5:$\approx$8.8:10 ratio of the relative amounts of $O^4$-EtThy, $O^2$-EtThy, and $O^6$-EtGua initially formed in EtNU-exposed cells (4, 49), the overproportional protective effect exerted by the selective repair of $O^6$-EtGua underscores the predominant role of the latter DNA lesion. Interestingly, when AI-clones originating from WT and $R^+$ variant cells previously exposed to EtNU were tested for tumorigenicity in syngeneic animals, the former produced highly malignant sarcomas in four of six cases, whereas only two of eight of the variant-derived AI-clones were tumorigenic, with slow-growing fibrosarcomas appearing after >3 times longer latency periods. This difference might result from an increased probability for the occurrence of multiple mutations required for the expression of fully malignant phenotypes in WT cells due to the persistence of $O^6$-EtGua in critical genes, and it suggests that acquisition of the capacity for anchorage-independent proliferation *in vitro* and tumorigenicity *in vivo* are not necessarily interdependent.

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