[CANCER RESEARCH 57, 581–584, February 15, 1997]

Advances in Brief

Relative Susceptibilities of *XPA* Knockout Mice and Their Heterozygous and Wild-Type Littermates to UVB-induced Skin Cancer[1]

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Abstract

Although xeroderma pigmentosum (XP) patients are rare, carriers of *XP* genes (heterozygotes) are much more common. Whether such carriers have an increased skin cancer risk is unknown. Recently developed mouse models for XP have opened up the possibility of determining the skin cancer risk of heterozygotes relative to wild types. Therefore, the *XPA* knockout trait has been crossed into hairless mice, and squamous cell carcinomas of the skin have been induced by low daily UVB exposures for 500 days in all three genotypes (−/−, +/−, and +/+). The carcinogenic response of the heterozygotes did not significantly differ from that of their wild-type littermates. Tumors in the *XPA* −/− animals appeared with a latency time that was decreased by a factor of 4.2. From this, we estimate that a functional *XPA* gene provides a "protection factor" of 60 (95% confidence interval, 15–250) against UV carcinogenesis, which is greater protection than that against acute UV effects, such as erythema and edema (protection factor between 7 and 16). Deficient nucleotide excision repair appears to have a more dramatic impact on skin cancer susceptibility than on sensitivity to acute UV effects.

Introduction

XP[3] is a genetic disease clinically characterized by an extremely high sensitivity to sunlight and risk of cutaneous cancers. At the molecular level, XP is characterized by defective nucleotide excision repair: cultured cells from XP patients are unable to remove UV-induced DNA damage from the genome, as measured by UDS. A subclassification of XP was established by cell fusion studies with cultured fibroblasts of different XP patients. Heterokaryons show either normal levels of UDS (patients belong to different complementation groups) or impaired levels of UDS (patients are in the same complementation group). Each complementation group represents a gene that causes XP if both alleles are dysfunctional. Thus far, seven complementation groups (XPA through XPG) have been identified in nucleotide excision repair-deficient XP (1). Although XP patients (homozygotes) are rare, carriers of *XP* genes (heterozygotes) are much more common in the general population (2). Whether such carriers have an increased skin cancer risk is unknown. Mouse models for XP can serve to answer this question (2).

Recently, the mouse homologue of the human genes responsible for XPA (3) and XPC (4) have been cloned, and subsequently, knockout mice have been generated (4–6). These mice were shown to have a phenotype comparable to XP patients, *i.e.*, an increased susceptibility to UV-induced skin cancer (4–6). An estimation of the magnitude of the effect could not be made from these studies because the duration of the experiments was too short for the development of tumors in the heterozygous and wild-type counterparts. We therefore carried out an experiment in which *XPA* −/− mice and their heterozygous and wild-type littermates were daily exposed to UV radiation for up to 500 days. This experiment provides information on the relative risk of heterozygotes and on the "protection factor" (analogous to the one stated on sunscreens) that functional *XPA* genes provide against the carcinogenic effects of UVB exposure.

Hitherto, UV carcinogenesis in mice with introduced DNA repair deficiencies has been carried out in animals that had to be shaved once or twice a week, as a dense hair coat is an efficient optical filter. There is a long-standing tradition of photocarcinogenesis in SKH hairless mice, which do not have to be shaved and therefore allow a very accurate dosimetry (7–9). We have crossed *XPA* knockout mice with SKH hairless mice and intercrossed the hairless offspring. Hairless *XPA* heterozygotes (*XPA* +/−) and their homozygous (*XPA* −/−) and wild-type (*XPA* +/+) littermates were daily exposed to a very low UVB dose: less than 15% of the dose required to induce erythema or edema in the wild-type animals. As a result of this low daily exposure regimen, skin tumors developed in *XPA* heterozygotes and wild-type mice after more than 300 days, which makes the assay sensitive enough to detect potential small differences in cancer development between the heterozygotes and wild types. No statistically significant increase in overall susceptibility to UVB-induced squamous cell carcinomas of the skin could be detected in heterozygotes in comparison to wild types, whereas tumors in the *XPA* −/− mice appeared with an approximately 4 times shorter latency time.

Materials and Methods

Animals and Irradiation Setup. Inactivation of the mouse *XPA* gene was accomplished by replacing exons 3 and 4 with a *neo* marker cassette, followed by homologous recombination in embryonic stem cells (5). *XPA* knockout mice in a 129/ola-C57B1/6 background were crossed with albino hairless mice (HRA/SKH). The offspring was backcrossed with HRA/SKH, and an intercross of the hairless offspring was used for chronic UV exposures. Animals were housed individually in macrolon type I cages. Standard mouse chow (Hope Farms RMH-B) and tap water were available *ad libitum*. The mice were kept at an ambient temperature of 25 ± 1°C. The room was illuminated with yellow fluorescent tubes (Philips TL40W/16) in a 12-h cycle (switched on at 6:00 a.m. and off at 6:00 p.m.). These lamps do not emit any measurable UV radiation. No daylight entered the room.

A schematic representation of the irradiation set-up is shown in Fig. 1*A*. In this setup, three fluorescent American Philips F40 sunlamps were mounted above the animal compartments (see Fig. 1*B* for spectrum). A wire mesh was mounted between the lamps and the animals to reduce the dose rate at mouse level. Fine tuning of the dose was accomplished by electronic dimmer circuits that controlled the radiant outputs of the lamps.

Formal permission for the experiments was granted by an ethical committee of Utrecht University, as required by Dutch law.

Sensitivity to Acute UV Effects and Choice of Dose Groups. We assessed the maximum daily dose that could be tolerated and would not induce acute effects, such as erythema and/or edema, in any of the three genotypes. It has been shown previously that *XPA* −/− mice develop an erythema at UVB doses that do not induce any visible effects in the wild-type counterparts (5). To get an impression of the magnitude of the increase in UVB sensitivity of the

Received 12/3/96; accepted 1/2/97.

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[1] Financed by Grants EV5V-CT91-0030 and ENV4-CT96-0172 from the Environment Program of the European Community.

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[3] The abbreviations used are: XP, xeroderma pigmentosum; UDS, unscheduled DNA synthesis; UVB, 280–315 nm; UVA, 315–400 nm; SCC, squamous cell carcinoma; t_{50}, median tumor induction time; CS, cockayne syndrome.

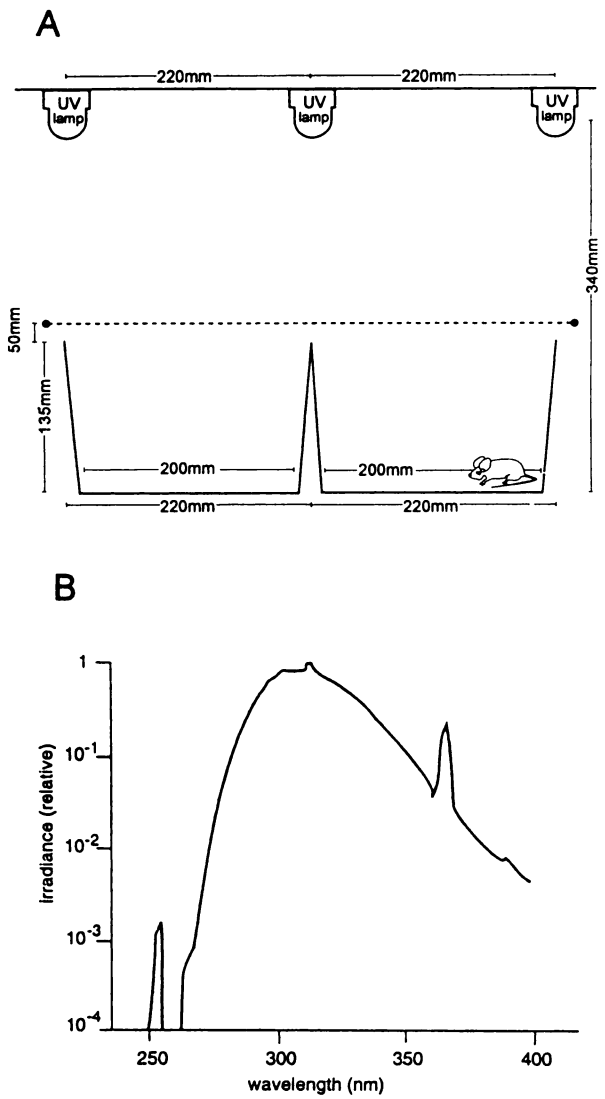

Fig. 1. *A*, scaled schematic representation of the exposure configuration. *B*, relative spectral energy output of the Philips F40 sunlamp.

XPA −/− mice, we exposed mice of each genotype to UVB radiation from a filtered Kromayer lamp. This lamp allows short erythema-inducing exposures to limited skin areas by placing the circular port (about 2.5 cm^2) of the source in close contact with the skin (10). *XPA* −/− mice were exposed for 1, 2, and 3 s and *XPA* +/− and *XPA* +/+ mice for 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, and 30 s of UV radiation from this lamp. All exposures were applied in duplicate on separate mice. The mice were checked for erythema and/or edema at 24, 48, and 80 h after the exposures.

The mice in the present experiment were an F2 intercross of the two parental strains, 129/ola-C57B16 and HRA/SKH. The most UV-sensitive parental strain is expected to be the SKH strain because these mice are albinos, whereas the 129/ola-C57B16 mice are pigmented. SKH mice tolerate a daily exposure of 1.3 kJ/m^2 (250–400 nm) UV radiation from F40 sunlamps, *i.e.*, they show no severe acute effects (11). From the differences in acute responses to the Kromayer (see "Results"), we estimated that 80 J/m^2 (1.3 kJ · m^{-2}/16) would be the highest daily dose tolerated by *XPA* −/− mice in the chronic exposure experiment. To determine the dose-response relationship for UV carcinogenesis in *XPA* knockout mice and to compare it with that for wild-type mice, another group of *XPA* −/− mice was daily exposed to 32 J/m^2. Each of the four groups (*XPA* −/−, *XPA* +/−, *XPA* +/+ at 80 J/m^2, and *XPA* −/− at 32 J/m^2) consisted of 14 mice, which were irradiated for 6 min/day, between 12:30 and 12:36 p.m. The dose rates were measured weekly and, if necessary, readjusted. In addition, control mice of all three genotypes were kept under the same circumstances, but not UV exposed.

Animal Observations, Definitions, and Data Analysis. Animals were checked weekly and all deviations from normal skin appearance (redness, scratching, hyperkeratosis, tumors, and so forth) were recorded. The tumor locations on the animal were mapped and numbered for each animal separately. If a lesion was spotted on an animal for the first time, the observation had to be confirmed in the next checkup or else the observation was disregarded. During the experiment, we distinguished between evident papillomas (pedunculated, protruding tumors with a "cauliflower-like" surface) and other tumors ("nonpapillomas") by visual inspection (8, 12). Tumors were also subdivided according to diameter: <1, ≥1, and ≥2 mm. Mice were sacrificed when they carried at least one tumor with a diameter ≥4 mm. The day of the first exposure of an experimental group was defined as $t = 0$. If a tumor was first seen at $t = t_i$, and $t = t_{i-1}$ was the previous checkup time, then the induction time was defined as $(t_i + t_{i-1})/2$. Graphical representation of the prevalence *versus* time is based on an actuarial method described by Kaplan and Meier (13), and adapted to carcinogenesis by Peto *et al.* (14). This procedure computes the chance of tumor-free survival. The death-corrected tumor prevalence is then given by 1 minus this chance. Prevalences, or times to first tumor, were fitted by a log-normal distribution using a maximum likelihood method for a concise description of the observations in terms of $\ln(t_{50})$ and the SD in ln(time to first tumor per mouse). The difference in tumor induction between the *XPA* +/− and the *XPA* +/+ group was tested by the nonparametric trend analysis as described by Peto *et al.* (14).

Results

Assessment of Acute Reactions. The maximum effect of the single exposures with the Kromayer lamp was seen at 48 h after exposure in all three genotypes. In the *XPA* −/− mice, only the exposures of 2 and 3 s caused an effect, and in the *XPA* +/− and the *XPA* +/+ mice, only the exposures of 16 s and longer caused an effect. Hence, the ratio in sensitivity to acute UVB effects of *XPA* −/− *versus* *XPA* +/−, +/+ is between 1:7 and 1:16.

Chronic Exposure Experiment. In the course of the chronic exposure experiment (500 days), nearly all animals contracted multiple skin tumors, whereas the unexposed control mice remained free of tumors. At the highest dosage, tumor development was preceded by redness, dryness, and scaling of the exposed dorsal skin in the *XPA* −/− mice, whereas at the low dose, as in the *XPA* +/− and +/+ mice, no other effects than tumor development were detected. In agreement with earlier UVB carcinogenesis experiments (7–9), only a very few frank papillomas arose in the wild-type and heterozygous mice; these benign tumors were excluded from further quantitative analysis. In the *XPA* −/− mice, hardly any papillomas were found in the highest dose group, whereas in the low dose group, 8 of 14 animals contracted one or more clear papillomas (see "Discussion"), which were also excluded from the quantitative data analysis.

Tumor Appearance in *XPA* +/− *versus* *XPA* +/+ Animals. The low daily UV exposure of 80 J/m^2 was chosen for two reasons. First, the increased UV sensitivity of the *XPA* −/− animals did not allow a higher daily exposure. Second, such a low daily exposure should make the experiment sensitive enough to detect potential small differences in skin cancer susceptibility between heterozygote and wild-type mice. If we plot the tumor prevalence on a probability scale *versus* time on a logarithmic scale, the points fall along a straight line: a cumulative log-normal distribution. Fig. 2 shows these plots and the log-normal distributions for the three genotypes and for two different tumor diameters, fitted according to a maximum likelihood method (13, 14). Table 1 gives the optimum values for the median tumor induction time (t_{50}), $\mu = \ln(t_{50})$, and the standard deviation (σ) of the fitted log-normal distributions. It can be seen that the prevalence curves run roughly parallel for the different genotypes (*i.e.*, σ values do not differ significantly). There was no statistically significant difference ($P > 0.05$) in tumor appearance between *XPA* +/− and *XPA* +/+ mice at any of the three tumor detection levels, except for the minimal tumors (diameter <1 mm), which just reach significance

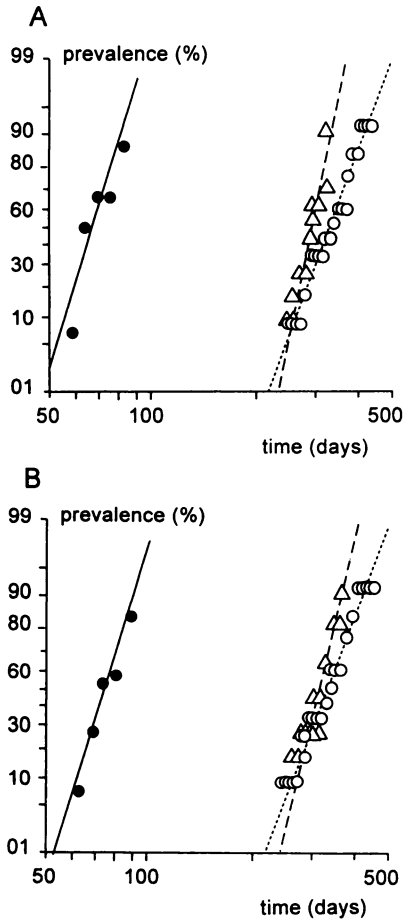

Fig. 2. Kaplan-Meier plot for the prevalence of tumors induced by daily exposure to 80 J/m^2 UV radiation for *XPA* −/− (●), *XPA* +/− (△), and *XPA* +/+ (○) animals. *A*, tumors <1 mm; *B*, tumors between 1 and 2 mm in diameter.

Table 1 *The log-normal parameters, μ [i.e., ln(t_{50} in days)] and σ, derived from the prevalence for different SCC diameters*

Group	<1 mm	≥1 mm	≥2 mm
XPA −/−, 32 J/m^2			
t_{50}	96	108	122
μ	4.56 ± 0.04	4.68 ± 0.04	4.80 ± 0.04
σ	0.14 ± 0.03	0.14 ± 0.03	0.13 ± 0.03
XPA −/−, 80 J/m^2			
t_{50}	69	78	90
μ	4.24 ± 0.04	4.36 ± 0.04	4.50 ± 0.04
σ	0.16 ± 0.03	0.15 ± 0.03	0.16 ± 0.03
XPA +/−, 80 J/m^2			
t_{50}	289	313	360
μ	5.66 ± 0.03	5.75 ± 0.04	5.89 ± 0.05
σ	0.10 ± 0.02	0.12 ± 0.03	0.17 ± 0.04
XPA +/+, 80 J/m^2			
t_{50}	308	331	360
μ	5.73 ± 0.04	5.80 ± 0.05	5.89 ± 0.07
σ	0.14 ± 0.03	0.18 ± 0.04	0.22 ± 0.05

only at a late stage in the experiment. This finding hinges on the last animal that got a minimal tumor in either one of the groups and is considered to be a spurious event because it is not reproduced at the other tumor detection levels.

UVB-induced tumors of hairless mice are almost exclusively SCCs or their precursors, carcinomas *in situ* (Bowen's disease) and actinic keratoses (7, 15). To check whether this holds for tumors on the heterozygotes, histological examination was performed on aselective samples of 12 tumors (≥2 mm) from heterozygous and 9 tumors (≥2 mm) from wild-type animals. By light microscopy, after H&E staining, 9 of the 12 tumors from heterozygotes were classified as SCCs, 2 as Bowen's disease, and 1 as actinic keratosis. Eight of the 9 tumors from wild-type mice were classified as SCCs and 1 as actinic keratosis.

Tumor Appearance in *XPA* −/− Mice at the Two Dose Levels. Fig. 3 shows that the prevalence curves of the *XPA* −/− mice run parallel for the two separate daily doses, and in Fig. 2 it is shown that they run parallel to those of the *XPA* +/− and *XPA* +/+ mice. Hence, the rate of development of tumors can be fully characterized by the t_{50} values. In Table 1, it is shown that the t_{50} values for the *XPA* −/− mice are drastically lower than those for *XPA* +/−, *XPA* +/+ animals at the same dose level. The average reduction in t_{50} for the *XPA* −/− mice *versus* *XPA* +/− and *XPA* +/+ equals a factor of 4.2 ± 0.2 (average for the two smallest tumor size categories).

For small tumors (≤1 mm), the relationship between t_{50} and daily dose can generally be described by:

$$t_{50} \propto D^{-r} \qquad \text{(A)}$$

where ∝ stands for direct proportionality, $r = 0.6$ (7, 16) for albino SKH mice, and $r = 0.3$ for pigmented SKH mice (16). We calculated the r value for the two *XPA* −/− dose groups. Using the data for the smallest tumors from Table 1, this yields $r = 0.35 \pm 0.06$, which does not differ significantly from earlier published data for pigmented mice (16).

Discussion

It has earlier been shown that mice with defective *XPA* genes have an increased sensitivity to UV-induced squamous cell carcinomas of the skin. The effect could not be quantified because in these experiments, only the knockout animals developed tumors, whereas the heterozygous and wild-type controls remained tumor-free. Concordantly, no conclu-

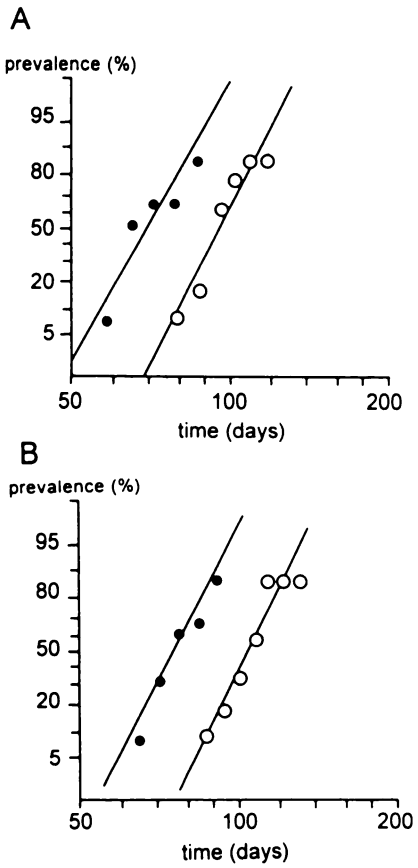

Fig. 3. Kaplan-Meier plot for the prevalence of tumors for *XPA* −/− mice induced by daily exposure to 80 (●) or 32 (○) J/m^2. *A*, tumors <1 mm; *B*, tumors between 1 and 2 mm in diameter.

sions could be drawn about a possible elevated skin cancer risk for the heterozygotes. In the present experiment, dosages and exposure times were sufficient to induce skin tumors in all three genotypes. We found that (*a*) heterozygotes do not have a higher susceptibility to UV-induced skin cancer than wild-type controls, and (*b*) *XPA*-deficient mice develop skin tumors with a latency time that is a factor of 4.2 ± 0.2 smaller than heterozygote and wild-type controls. The difference in skin cancer risk between −/− animals and +/−, +/+ animals can roughly be estimated from Fig. 2. If we extrapolate the prevalence curves of the +/−, +/+ animals to a timepoint <100 days, this prevalence can be compared with the one measured for the −/− animals. Such an extrapolation would yield a skin cancer risk for the *XPA* −/− mice that is $>10^4$ larger than that for the *XPA* +/−, +/+ mice, which is in line with data for human XP (17).

Functional *XPA* genes provide protection by efficient removal of the UVB-induced DNA damage, mainly cyclobutane pyrimidine dimers and [6-4] photoproducts, that is caused by the Kromayer lamp or the sunlamps. We, therefore, expect a similar protection factor of the *XPA* gene for both types of lamps. From experiments with the Kromayer lamp on acute sensitivity, we found that functional *XPA* genes appeared to decrease the acute UV sensitivity by a factor of 7–16. For the tumor induction experiment with the F40 sunlamps, the protection factor had to be estimated indirectly. Using Eq. A, we can determine the protection factor of a functional *XPA* gene, *i.e.*, calculate which decrease in dose increases t_{50} by a factor of 4.2. Using $r = 0.35 \pm 0.06$, we found a dose decreased by a factor of 60 (95% confidence interval, 15–250). It is evident that the reliability of this protection factor strongly depends on an accurate determination of r, which requires a large-scale animal experiment comprising more dose groups. Despite the large inaccuracy in its determination, we can conclude that the protection factor of a functional *XPA* gene against UVB-induced skin cancer is substantially larger than that against UVB-induced erythema and edema (which falls between 7 and 16). This indicates that equally erythemogenic exposures of *XPA* −/− mice and *XPA* +/+ mice will result in earlier tumor appearance in the −/− animals than in the +/+ animals. Deficient nucleotide excision repair appears to have a less dramatic effect on sensitivity to acute UV effects than on skin cancer susceptibility.

Hairless mice under chronic UVB exposure only incidentally develop a papilloma, whereas under UVA exposure, both papillomas and SCCs occur (7–9, 12). To our surprise, a substantial number of *XPA* −/− mice in the low-dose group developed one or more papillomas in the present UVB experiment, whereas in the high-dose group, hardly any papillomas were encountered. A possible explanation for this observation could be that the induction of papillomas in these mice has a dose dependence less steep than that of the induction of SCCs. This implies that in the high-dose group, the SCCs in the *XPA* −/− mice occurred well before papillomas had time to appear, whereas in the low-dose group, the papilloma development was slowed down less than that of SCCs so that the order of occurrences was reversed.

In the present experiment, it has been shown that the introduction of a complete deficiency in nucleotide excision repair in mice leads to a speeding up of tumor development by a factor of 4.2, which corresponds to an increase in susceptibility to a carcinogenic UV dose by a factor of 60 (95% confidence interval, 15–250). Nucleotide excision repair operates with variable efficiencies in different parts of the genome (18). DNA strands transcribed by RNA polymerase II are repaired rapidly (transcription-coupled repair), whereas the bulk of the DNA is repaired less efficiently (global genome repair). Genetic diseases with deficiencies in only transcription-coupled repair or only global genome repair are CSB (19) and XPC (20), respectively. *XPC* knockout mice have been described (4) and *CSB* knockout mice have been established very recently.[4] Both *XPC* −/− (4) and *CSB* −/− mice[4] appeared to have a higher susceptibility to UVB-induced skin cancer than their wild-type counterparts. Carrying out experiments with *CSB* and *XPC* knockout mice similar to the present experiment with *XPA* knockouts will allow us to unravel the relative importance of transcription-coupled repair *versus* global genome repair in UV carcinogenesis.

Acknowledgments

We thank H. Sturkenboom for maintenance of the animals, K. Guikers for writing computer programs for the data processing, and Dr. J. Toonstra for histological characterization of the tumors.

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Cancer Res 1997;57:581-584.