REPORTS

Diagnostic Testing Following Screening Mammography in the Elderly

H. Gilbert Welch, Elliott S. Fisher

Background: **To provide some sense of the general frequency and timing of diagnostic testing following screening mammography in the United States, we investigated the experience of women screened in the Medicare population.** ***Methods:*** **By use of Medicare's National Claims History System, we identified a cohort (n = 23 172) of women 65 years old or older screened during the period from January 1, 1995, through April 30, 1995, and tracked each woman over the subsequent 8 months for the performance of additional breast imaging and biopsy procedures. Using two claims-based definitions for newly detected breast cancer, we also estimated the positive predictive value of screening mammography.** ***Results:*** **For every 1000 women aged 65–69 years who underwent screening, 85 (95% confidence interval [CI] = 79–91) had follow-up testing in the subsequent 8 months; 76 (95% CI = 71–82) had additional breast imaging, and 23 (95% CI = 20–26) had biopsy procedures. Corresponding numbers for women aged 70 years or more were similar. Some women underwent repeated examinations; 13% of those receiving diagnostic mammograms had more than one; 11% of those undergoing biopsy procedures had more than one. About half of the women who underwent a biopsy had the procedure more than 3 weeks after the imaging test upon which the decision to perform a biopsy was presumably made. The estimated positive predictive value of an abnormal screening mammogram (defined as a mammogram that engendered additional testing) was 0.08 (95% CI = 0.06–0.10) for women aged 65–69 years and 0.14 (95% CI = 0.12–0.16) for women aged 70 years or more.** ***Conclusion:*** **Additional testing is a frequent consequence of screening mammography and may require a considerable period of time to come to closure. The need for additional testing, however, is weakly predictive of cancer. [J Natl Cancer Inst 1998;90:1389–92]**

No single outcome can capture the net effect of screening *(1).* Any reduction in mortality among those who have the target disorder must be balanced against the cascade of subsequent testing for those who do not. The balance is affected by the threshold for subsequent testing: If too few receive subsequent testing, then there can be little early diagnosis and little benefit; if too many do, then any benefits of earlier diagnosis may be overwhelmed by the anxiety, unnecessary diagnosis, and unnecessary treatment associated with excessive testing. Data on downstream testing are important not only to help assess the net effect of screening, but also to help clinicians inform and prepare their patients *(2).*

Few screening tests have been as thoroughly studied as mammography. In the past 30 years, nearly a half million women have participated in randomized trials designed to quantify the mortality benefit. But testing following screening mammography is less well studied. Published studies represent the experience of select mobile mammography programs *(3,4),* private radiology groups *(5,6),* or different countries *(7,8).* Because these data may not be representative of general practice in the United States, we investigated diagnostic testing received by Medicare beneficiaries following a single screening mammogram.

Subjects and Methods

Data

As part of our work on the *Dartmouth Atlas of Health Care (9),* we used 1995 data from Medicare's National Claims History System (Source: Health Care Financing Administration, Baltimore, MD) to study women who received mammography. These files contain all physician claims for a 5% sample of Medicare Part B beneficiaries and include both the Current Procedure Terminology (CPT) code and the date of service. Because individuals who become eligible for Medicare for reasons other than age represent a unique group, we excluded the few beneficiaries who were under age 65. In addition, we excluded beneficiaries enrolled in risk-contract managed care plans (<8% of the sample) because diagnostic services provided to them are not reported in the claims.

Screening Mammography

Conceptually, screening mammography implies a bilateral mammographic examination performed on a woman who has neither a palpable mass nor symptoms of breast cancer. In 1991, Medicare began to allow payment for one screening mammographic examination every 2 years. Although there is a CPT code specifically identifying screening mammography (CPT code 76092), over half of all bilateral mammograms paid for by Medicare are coded using an ostensibly ''diagnostic'' code: bilateral mammography (CPT code 76091). Given this volume, many screening films are undoubtedly miscoded as diagnostic films (probably a reflection of both historical practice and payment policy). It is difficult, therefore, to determine precisely what proportions of women are genuinely ''screened.''

Consequently, we calculated the proportion of women receiving mammography during calendar year 1995 by using two methods (Table 1). For the lower bound calculation, the numerator was the number of women receiving screening mammography (CPT code 76092); for the upper bound calculation, the numerator was the number of women receiving any bilateral mammography (either CPT code 76091 or CPT code 76092). The denominator was the same for both calculations: female Medicare beneficiaries 65 years old or older who were enrolled in Part B, who were not in risk-contract managed care plans, and who were alive at the end of 1995 *(10).*

Downstream Events

For our analysis of diagnostic testing following screening mammography, we wanted to focus on the experience of women being screened; thus, we used the more targeted code. The remaining data presented (Table 2; Figs. 1 and 2) are based on a cohort of 23 172 women who received a screening

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Table 1. Proportion of female Medicare beneficiaries in various age groups receiving a screening mammogram and any bilateral mammogram in calendar year 1995*

	% of women receiving	
Age group, y	Screening mammography	Any bilateral mammography
65–69	13.9	29.9
70–74	11.7	26.5
75–79	9.4	21.2
80–84	6.4	14.2
85–89	3.5	7.9
90–94	1.4	3.2
⩾95	0.4	0.9

*Medicare reimbursement regulations allow only screening mammography biennially.

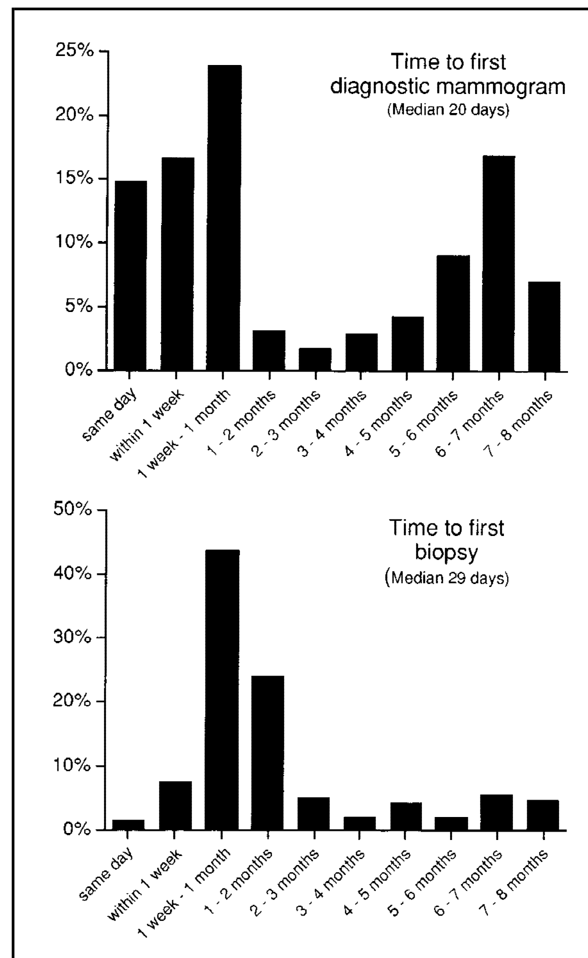

Fig. 1. Time interval between screening mammography and subsequent testing. Results are based on 1395 women receiving diagnostic mammograms and 573 undergoing biopsy.

mammographic examination (CPT code 76092) during the period from January 1, 1995, through April 30, 1995.

We tracked each woman in the cohort for 8 months following her index screening mammogram. We sought two broad categories of subsequent utilization: breast imaging and biopsy procedures. Breast imaging included subsequent diagnostic mammograms (unilateral and bilateral) and breast ultrasound (the code for breast magnetic resonance imaging was not yet in use). Biopsy procedures included three methods for obtaining pathologic specimens (fine-needle aspiration, needle core biopsy, and excisional biopsy) and two imaging procedures used to assist the process (needle localization and stereotactic localization).

Positive Predictive Value

To calculate the positive predictive value (the probability of breast cancer, given an abnormal mammogram), we needed to define both a new case of breast cancer (the numerator) and an abnormal mammographic examination (the denominator). Our definition of an abnormal mammogram is similar to that used by Kerlikowske et al. *(4):* one that necessitates further diagnostic testing.

We developed two claims-based definitions to identify new cases of breast cancer. The finding of a diagnostic code for breast cancer by itself is insufficient because it does not necessarily reflect a new case—women with previously treated breast cancer appropriately undergo screening mammography. For our less stringent definition of cancer (i.e., identifying more cases), a new case of cancer was defined as any case in which a diagnostic code for breast cancer first appeared after a biopsy. Because screening mammography and biopsy could appear in our data before an office visit (with which diagnostic codes most reliably appear), the foregoing definition still includes some women with pre-existing cancer who undergo biopsy (yet in whom no new case of cancer was found).

For our more stringent definition, therefore, women were considered to have breast cancer only if they received treatment. Breast cancer treatment included any one of the following: surgery (i.e., mastectomy, lumpectomy, or axillary dissection), radiation therapy, or chemotherapy. Given that this was a screening cohort (i.e., one in which treatment would be contemplated), we believe that this definition most closely approximates new cases of cancer.

Table 2. Diagnostic testing per 1000 women in the 8 months following screening mammography

	No. of women undergoing test per 1000 screened (95% CI)*	
	Ages 65–69 y	Ages ⩾70 y
Any additional imaging	76 (71–82)	70 (66–74)
Diagnostic mammogram	64 (58–69)	58 (55–62)
Breast ultrasound	24 (21–28)	21 (19–23)
Any biopsy procedure	23 (20–26)	26 (23–29)
Fine-needle aspiration	4 (3–6)	5 (4–6)
Needle core	4 (3–6)	4 (3–5)
Excisional biopsy	15 (13–18)	19 (16–21)
Needle localization	12 (10–15)	15 (13–17)
Stereotactic localization	3 (2–4)	2 (1–3)
Abnormal screening mammogram (any additional imaging or biopsy)	85 (79–91)	81 (77–86)
Cases of breast cancer	7 (5–8)	11 (10–13)
Positive predictive value	0.08 (0.06–0.10)	0.14 (0.12–0.16)

*Based on sample numbers of 8548 and 14 624, respectively; CI = confidence interval.

RESULTS

Table 1 shows the proportion of women receiving either screening mammography or any bilateral mammography in 1995. As has been noted for earlier years *(11)*, the proportion of the Medicare population being screened remains small. Fewer than a third of beneficiaries in the target age group (under age 70 years) *(12)* are being regularly screened, even with the use of the most generous calculation. On the other hand, Table 1 also shows that some women continue to receive screening mammography well into old age (50 000 women 85 years old or older were screened), despite its marginal impact on life expectancy *(13)*.

Table 2 delineates our primary results:

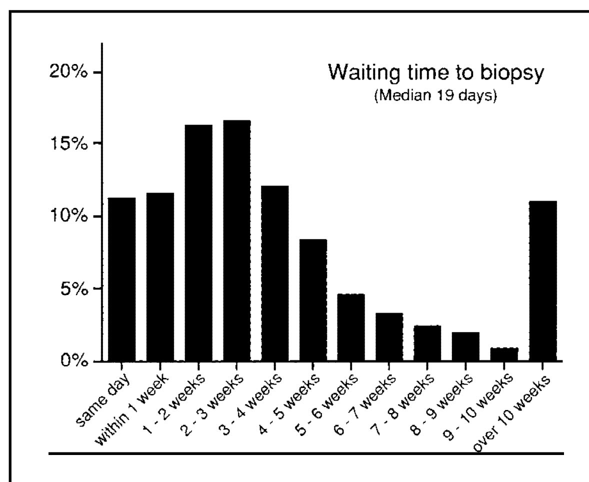

Fig. 2. Distribution of waiting times for 573 women undergoing biopsy. Waiting time was measured from the imaging test that most immediately preceded the biopsy (presumably the test on which the decision to biopsy was based).

the number of women undergoing various diagnostic tests per 1000 screened. The results here are divided into two age groups: ages 65–69 years [those for whom screening mammography is currently recommended *(12)*] and ages 70 years old or older (those for whom there is no recommendation). For both groups, the most common test following screening mammography is diagnostic mammography (64 per 1000 and 58 per 1000, respectively). The next most common tests are breast ultrasound and some form of biopsy. Each occurs in roughly 25 women per 1000 screened, regardless of age. The total number of women receiving any additional testing (our definition of abnormal mammogram) was also similar in the two age groups (85 per 1000 and 81 per 1000, respectively).

As would be expected, the estimated number of cases of cancer (and, thus, positive predictive value) did rise with age. We used two claims-based definitions of cancer (*see* ''Subjects and Methods'' section). Both approaches yielded the same result for women aged 65–69 years: seven cancer cases per 1000. For women 70 years of age or older, our more stringent definition identified 10 cases per 1000, whereas our less stringent definition identified 11 cases per 1000. With the use of the latter estimate (leading to a higher positive predictive value), the positive predictive value of an abnormal mammographic examination was 0.08 (95% confidence interval [CI] = 0.06–0.10) for women aged 65–69 years and 0.14 (95% CI = 0.12–0.16) for women 70 years of age or older.

Fig. 1 depicts the time interval between screening mammography and two subsequent tests: diagnostic mammography and biopsy. Some women, however, underwent these examinations more than once (i.e., occurring on distinct days): 13% of those receiving diagnostic mammograms had more than one; 11% of those receiving biopsies had more than one. Both graphs in Fig. 1 reflect the time interval to the first examination.

As shown in the top graph of Fig. 1, 15% of women receiving diagnostic mammograms did so on the same day that they received their screening mammogram. Another 40% had their first diagnostic mammographic examination within a month. These two groups most likely represent those women deemed to require additional views on the basis of their screening mammogram. On the other hand, 40% had a diagnostic mammogram in 4–8 months, most likely representing a ''6-month'' follow-up film.

The course for women who undergo biopsy is different. As shown in the bottom graph of Fig. 1, most women receive the biopsy procedure within the first 2 months of the screening mammogram. This does not necessarily reflect the waiting time for biopsy, however, because about half of the women who ultimately underwent a biopsy procedure in our cohort also received intermediate testing (i.e., diagnostic mammography and/or breast ultrasound). To gauge the waiting time, we calculated the time between the first biopsy and the imaging test that most immediately preceded it (i.e., the test upon which the decision to perform a biopsy was presumably made). As shown in Fig. 2, almost half of women waited more than 3 weeks.

Discussion

Eight major randomized trials have produced a reasonably consistent estimate of the reduction in mortality afforded by screening women aged 50–70 years: a 30%–40% reduction in breast cancer mortality *(14,15)*. Assuming these data pertain to actual practice in the United States, the average 65-year-old woman undergoing regular screening could expect a decline in annual breast cancer mortality from about one per 1000 *(16)* to 0.6–0.7 per 1000. This very real mortality benefit must be balanced against the chances that, through screening, she will learn that her breasts may be somehow abnormal, which will occur about 85 times per 1000 women screened.

There are several limitations to the claims data. First, it is reasonable to ask whether our data reflect a true screening cohort or include women with palpable breast lesions. The latter group might be classified as ''symptomatic'' and thus not eligible for screening. The occurrence of fine-needle aspiration and needle core biopsy, in particular, suggests that some patients may have had palpable breast lesions previously detected by clinical breast examination. On the other hand, the presence of a palpable lesion may have been discovered only *after* the screening mammogram. In fact, the reported experience is that screening mammography routinely identifies patients who are subsequently noted to have palpable lesions *(4,8,17–19)*.

Nevertheless, we wanted to specifically consider the possibility that some diagnostic mammograms might be misclassified as screening mammograms. Although the high volume of women undergoing ''diagnostic'' mammography suggests that screening mammograms are sometimes miscoded as diagnostic, we found little evidence that miscoding occurs in the other direction (i.e., only 12 claims for second ''screening'' mammograms occurred in our cohort of 23 172). Furthermore, in a separate analysis of women undergoing diagnostic mammography during the same time period, we found that the numbers of women undergoing additional imaging (120 per 1000) and biopsy (55 per 1000) were substantially higher than those reported here. These findings, combined with an exceptionally low mortality in the sample of

women who underwent screening mammography (approximately 1% died in 1995), lead us to believe that this is genuinely a screening cohort.

Another limitation is that our cohort is undoubtedly a mixture of women receiving their first screening mammogram and women receiving a repeat examination. It is well established that more follow-up testing occurs after first examinations than after repeat examinations *(4,7)*. Our cohort had higher rates of subsequent testing than those reported for similarly aged women undergoing their first examination in San Francisco, CA *(4),* and the rates in our cohort were substantially higher than those reported in Sweden *(18,19)*. That our cohort is a mixture of first and repeat examinations suggests that the typical practice pattern in the United States involves lower thresholds for subsequent testing than previously reported.

Finally, a number of questions might be raised about our calculation of the positive predictive value. Our claims-based definition would have undercounted cases of cancer (leading to an underestimate of the positive predictive value) if women were lost to follow-up or chose not to undergo therapy. We have evidence that loss to follow-up was a relatively small problem (<1% received no medical services in the 8 months following screening mammography). Although women might choose to forego therapy (probably unusual in the setting of screening), to be missed in the claims they would also have to have had no diagnosis of breast cancer coded on subsequent visits. On the other hand, we may have undercounted the number of abnormal mammograms (leading to an overestimate of the positive predictive value) because we required that additional testing actually take place (not just be recommended). For perspective, our positive predictive values (0.08 in women aged 65–69 years and 0.14 for women aged 70 years or more) are similar to those reported from Florence, Italy (0.08 in women aged 60–69 years) and lower than those reported from San Francisco (0.17 in women aged 60–69 years and 0.19 for those aged 70 years or more) *(4)*.

The net benefit of screening mammography in practice depends on the intensity of the screening process. Our data suggest that general practice in the United States may involve lower thresholds for subsequent testing for older women than have been described previously. Furthermore, in delineating the timing of such testing, these data help define the period of time that women are exposed to diagnostic uncertainty—uncertainty that may cause considerable anxiety *(20,21)*. The data say nothing, however, about the reassurance of a normal examination. We hope that our findings help clinicians and their older patients make an informed decision about screening mammography.

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Notes

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Benzo[*a*]pyrene Diol Epoxide and Bleomycin Sensitivity and Susceptibility to Cancer of Upper Aerodigestive Tract

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Background: **Tobacco smoking is an established risk factor for cancers of the upper aerodigestive tract, and measurement of chromosomal aberrations, i.e., chromatid breaks, induced in lymphocytes *in vitro* by bleomycin has been shown to be a predictor of risk for these cancers. In a case–control study, we recruited case subjects who were previously treated with surgery and/or radiotherapy for stage I or stage II squamous cell carcinoma of the head and neck to test the hypothesis that lymphocytic chromatid breaks induced by benzo[*a*]pyrene diol epoxide (BPDE), a tobacco mutagen, may also be associated with risk of developing cancers of the upper aerodigestive tract.** ***Methods:*** **Case subjects were matched to control subjects on the basis of age, sex, ethnicity, and smoking status. Primary lymphocytes from 67 case subjects and 81 control subjects were treated with 2 μ*M* BPDE for 24 hours, and the frequency of induced chromatid breaks was determined. All statistical tests were two-sided.** ***Results:*** **Lymphocytes from case subjects compared with lymphocytes from control subjects showed significantly more breaks per cell induced by BPDE (mean ± standard deviation, 0.77 ± 0.38 versus 0.49 ± 0.25; *P*<.001). Lymphocytes from 64.2% of case subjects were sensitive to BPDE (using a cutoff value of ⩾0.60 break per cell). Subjects in the highest quartile of chromatid breaks had an approximately 20-fold increased risk of cancer compared with those in the lowest quartile after adjustment for age, sex, ethnicity, and smoking status. The association between BPDE sensitivity and cancer risk was higher in former smokers than in current smokers and higher in younger patients than in older patients. Subjects with sensitivity to both BPDE and bleomycin were at a 19.2-fold increased risk of cancer compared with those who were not sensitive to either agent.** ***Conclusions:*** **Mutagen sensitivity assays may aid in identifying individuals at risk of cancer, and use of parallel assays with two mutagens may improve risk predictability. [J Natl Cancer Inst 1998;90:1393–9]**

Cancers of the upper aerodigestive tract accounted for 3.0% of all incident cancers and 2.3% of all fatal cancers in the United States in 1997 *(1).* These percentages translate into 41 650 new case subjects and 12 670 deaths annually *(1).* Like lung cancer, cancers of the upper aerodigestive tract are paradigms of environmentally induced diseases. Although an interaction of host susceptibility to cancer and environmental exposure to carcinogens is well established in lung cancer, there are less data on susceptibility to cancer in patients with cancer of the upper aerodigestive tract.

Individual responses to environmental mutagens are highly variable, probably because of interindividual variations in the metabolism of carcinogens, susceptibility to chromosome damage in response to mutagens or carcinogens, and/or differing DNA repair capacity *(2–8).* Hsu *(8)* suggested that susceptibility to chromosome damage varies along a continuum, with the recognized chromosome fragility syndromes such as Fanconi's anemia or ataxia-telangiectasia at the extreme of the spectrum. He hypothesized that genetic damage accumulates more rapidly in individuals with an inherent susceptibility to DNA damage and that those individuals might therefore be at higher risk for neoplastic cell transformation *(8).*

To substantiate this hypothesis, Hsu et al. *(9)* developed the bleomycin sensitivity assay, in which the number of chromatid breaks induced by *in vitro* exposure to bleomycin (a radiomimetic mutagen) in short-term cultures of peripheral blood lymphocytes is assessed to gauge host susceptibility. Spitz et al. *(10–12)* have shown that bleomycin sensitivity is an independent predictor of risk of developing cancer of the upper aerodigestive tract and that the phenotype is constitutional.

Different carcinogens or mutagens may act on cells through different molecular mechanisms and, therefore, may activate different repair pathways. A person who is sensitive to one mutagen may be resistant to another *(13).* A battery of assays utilizing different mutagens may be necessary to adequately characterize cancer risk *(14).* Bleomycin causes oxidative damage and single- and double-strand breaks requiring base-excision repair and the action of polymerases. In tobacco smoke, various compounds may generate DNA damage through oxidative damage. However, upon interaction with DNA, benzo[*a*]pyrene diol epoxide (BPDE), the metabolic product of benzo[*a*]pyrene, a constituent of tobacco smoke, forms covalent adducts that are repaired through the nucleotide excision pathway. We therefore selected BPDE as the test mutagen, hypothesizing that it is the more relevant mutagen for studying tobacco-related cancers, such as cancers of the upper aerodigestive tract, and that using bleomycin and BPDE in parallel assays might improve risk prediction.

Subjects and Methods

Study Subjects

The subjects recruited by our study were derived from an ongoing chemoprevention study. Case subjects were eligible for the interventional study if they had a confirmed diagnosis of stage I or stage II squamous cell carcinoma of the upper aerodigestive tract (oral cavity, pharynx, or larynx) as defined by the American Joint Committee on Cancer staging criteria *(15)* and if the diagnosis had been made within the past 3 years. Most case subjects had been

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treated previously with surgery and/or radiotherapy but were free of cancer at the time of enrollment. The case subjects were identified from The University of Texas M. D. Anderson Cancer Center, from its Community Clinical Oncology Program (CCOP)[1] affiliates, and from Radiation Therapy Oncology Group (RTOG) centers throughout the country. These data are derived from baseline examinations for entry into the trial during the period from November 1991 through July 1995. Blood samples were obtained from participants before random assignment into the chemoprevention program. The case subjects in this report are patients for whom baseline blood samples were available for performing the mutagen sensitivity assay and are derived from the patient population that has formed the basis of two previous reports *(16,17)*.

Control subjects without cancer were selected from a comparison population being ascertained for two ongoing case–control studies of lung cancer. This is a collaboration with the largest health maintenance organization in the Houston metropolitan area (Kelsey Seybold Clinics) to identify, by survey distribution at clinic sites, a pool of potential control subjects characterized by age, sex, ethnicity, smoking status, and willingness to participate in these studies *(18)*. This is an excellent example of promoting research in a managed care environment. Control subjects were selected so as to match the case subjects on the basis of age (±5 years), sex, ethnicity, and smoking status (current, former, and never).

This research was approved by all relevant review boards and in accord with an assurance filed with and approved by the U.S. Department of Health and Human Services. Signed informed consent forms were obtained from the study subjects.

Data Collection

Epidemiologic data were collected from questionnaires that comprehensively elicited recent and not so recent histories of cigarette smoking and alcohol consumption. Blood was drawn into sodium-heparinized tubes for cytogenetic and molecular genetic analyses. The personnel who performed the laboratory analyses were blinded with regard to patient and control status.

Chromosome Analyses

A fresh whole blood sample (1 mL) was diluted with 9 mL of RPMI-1640 medium (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD) supplemented with 20% fetal bovine serum (Life Technologies, Inc.), 2 m*M* L-glutamine (Life Technologies, Inc.), 50 U/mL penicillin (Life Technologies, Inc.), 100 μg/mL streptomycin (Life Technologies, Inc.), and phytohemagglutinin (final concentration of 0.11 mg/mL) (Wellcome Research Laboratories, Research Triangle, NC). The cultures were incubated at 37 °C with 5% CO_2 for 72 hours. For the BPDE sensitivity assay, 40 μL of 0.5 m*M* BPDE (benzo[*a*]pyrene-γ-7, t-8-dihydrodiol-t-9, 10-epoxide [+/−] [anti]; Midwest Research Institute, Kansas City, MO) was added to each culture (final concentration, 2 μ*M*), and treatment was continued for 24 hours. We used tetrahydrofuran (Sigma Chemical Co., St. Louis, MO) as the solvent for BPDE. The stock solution (concentration of 1 m*M*) of BPDE in tetrahydrofuran was aliquoted into microcentrifuge tubes (500 μL each) and stored at −20 °C in the dark. The average number of breaks per cell by tetrahydrofuran alone at 40 μL/10-mL blood culture was 0.06. We used the established protocol *(9)* for the bleomycin sensitivity assay. In this assay, bleomycin (Nippon Kayaku Co., Ltd., Tokyo, Japan) was added to each culture to a final concentration of 0.03 U/mL, and treatment lasted for 5 hours. During the last hour, the cells were treated with 0.04 μg/mL Colcemid to arrest them in mitosis before they were harvested for conventional air-dried preparations. All prepared slides were coded and stained with Giemsa. The numbers of breaks were counted in 50 metaphases per sample, and the findings were expressed as the mean number of breaks per cell. We recorded only frank chromatid breaks or exchanges, not chromatid gaps or attenuated regions. When the length of an attenuated region was shorter than the width of the chromatid, it was diagnosed as a gap. If the length of the attenuated region was equal to or longer than the diameter of the chromatid, it was called a break. The number of breaks in one cell ranges from 0 to 12. If a cell had more than 12 breaks, that cell was excluded. However, such cells are very rare. Lee et al. *(19)* have shown that counting 50 cells yields sufficient validity and reliability. The distribution of the numbers of breaks in each cell follows a negative binomial distribution that can be considered as a gamma mixture of the Poisson distribution in the following sense: If the breaks per cell for each cell follow a Poisson distribution while the parameter of the Poisson distribution follows a gamma distribution, the breaks per cell in the whole group will follow a negative binomial distribution *(19)*. All the slides were coded; therefore, the laboratory personnel could evaluate them without knowledge of the case–control status.

Statistical Analysis

Student's *t* test was used to analyze the subject's age, the number of cigarettes smoked, the number of pack-years, BPDE sensitivity, and bleomycin sensitivity as continuous variables. These were also analyzed as categorical variables and dichotomized at the median value of the control group. We used 65 years to classify younger and older age groups and used 40 or more pack-years, 34 or more years of smoking, or 30 or more cigarettes smoked per day as cutoff points to stratify light and heavy smokers. The sensitivities to BPDE and bleomycin were considered positive if the values of breaks per cell were equal to or larger than the 75th percentile value of breaks per cell in the respective control groups (0.60 break per cell was the cutoff point for BPDE sensitivity, and 0.70 break per cell was the cutoff point for bleomycin sensitivity). We also repeated the analyses using other cutoff points and found that the results did not change substantially for either of the mutagens tested. A smoker was defined as a person who had smoked 100 cigarettes in his or her lifetime. A former smoker was defined as a person who had not smoked for at least 1 year. The associations among mutagen sensitivity, tobacco use, and cancer risk were estimated by univariate odds ratios (ORs). Woolf's method *(20)* was used to compute the 95% confidence intervals (CIs). A simple, stratified analysis was used to test for interactions between variables. To remove the confounding effects of other factors, we performed multiple logistic regression analysis using STATA statistical software *(21)*. All statistical tests were two-sided.

In this study, parametric and nonparametric bootstrap samplings were performed to evaluate the validity of the model and to assess the sensitivity of the cutoff selection with the use of the 75th percentile value in the control group. The bootstrap method creates pseudo-datasets by sampling observations with replacement from each within-stratum pool of observations. An entire dataset is thus created, and *P* values for all tests are computed on this pseudo-dataset. A counter is set up for each individual test to record whether the minimum *P* value from the pseudo-dataset is less than or equal to the actual *P* value for each base test. This process is repeated many times, and the proportions of occurrences of the minimum pseudo-*P* values being less than or equal to each of the actual *P* values are the adjusted *P* value reported by PROCMULTTEST. Bootstrap CIs were computed on the basis of the percentile method and the biased correction and accelerated method. All bootstrap analyses were based on 5000 bootstrap samples *(22)*.

RESULTS

The characteristics of the case subjects and control subjects are summarized in Table 1. There were 67 case subjects and 81 control subjects. Of the 67 case subjects, 55 were men and 12 were women, 61 were white and six were African-American, and six were never smokers, 31 were former smokers, and 30 were current smokers. Of the 81 control subjects, 64 were men and 17 were women, 75 were white and six were African-American, and six were never smokers, 49 were former smokers, and 26 were current smokers. The smoking status of the case subjects and control subjects did not differ significantly. The mean age was 62.2 years for the case subjects and 62.9 years for the control subjects. The mean pack-years was 44.4 for the case subjects and 48.4 for the control subjects, but this difference was not statistically significant. There was one subject with missing pack-year information. In terms of stage, 45 case subjects had stage I disease and 22 had stage II disease. Information on prior treatment was available for 63 case subjects. Of these case subjects, 45 were treated with surgery alone, 12 were given radiotherapy, and six were treated with both surgery and radiotherapy.

The distribution of BPDE-induced breaks per cell in the case subjects and control subjects is summarized in Table 1. The mean number ± standard deviation (SD) of BPDE-induced breaks per cell was significantly higher in the case subjects with cancer of the upper aerodiges-

Table 1. Distribution of select host characteristics of control subjects and case patients with cancer of the upper aerodigestive tract

	Case subjects (n = 67)	Control subjects (n = 81)	Two-sided *P*
Sex			
Male, No. (%)	55 (82.1)	64 (79.0)	
Female, No. (%)	12 (17.9)	17 (21.0)	.639*
Ethnicity			
White, No. (%)	61 (91.0)	75 (92.6)	
African-American, No. (%)	6 (9.0)	6 (7.4)	.731*
Mean age, y (standard deviation)	62.2 (10.9)	62.9 (9.2)	.659*
Smoking status			
Never, No. (%)	6 (8.9)	6 (7.4)	
Former,† No. (%)	31 (46.3)	49 (60.5)	
Current, No. (%)	30 (44.8)	26 (32.1)	.219*
Mean pack-years (standard deviation)	44.4 (39.4)	48.4 (37.3)	.527*
Distribution of benzo[*a*]pyrene diol epoxide sensitivity	Breaks per cell		
Minimum	0.26	0.08	<.0001‡
1st quartile	0.51	0.32	
Median	0.68	0.46	
3rd quartile	0.95	0.60	
Maximum	2.24	1.42	
Mean (standard deviation)	0.77 (0.38)	0.49 (0.25)	
No. of cells per 50 cells showing chromatid breaks			
Range (low to high)	7–37	3–32	
Mean (standard deviation)	18.45 (5.81)	14.80 (5.71)	.0002§

**P* values are based on chi-squared test.
†Former smoker is defined as a smoker who had not smoked for at least a year.
‡*P* value is based on two-sided Wilcoxon rank sum test.
§*P* value is based on Student's *t* test.

tive tract (0.77 ± 0.38) than in the control subjects (0.49 ± 0.25) (Student's *t* test, $P<.001$). The mean number of cells showing chromatid breaks per 50 metaphases was significantly higher in case subjects (18.45 ± 5.81) than in control subjects (14.80 ± 5.71) ($P = .0002$), ranging from seven to 37 cells for case subjects and three to 32 cells for control subjects. Upon univariate analysis, with the use of the 75th percentile value of the number of breaks per cell in the control subjects (i.e., 0.60 break per cell) as the cutoff point for BPDE sensitivity, mutagen sensitivity was associated with a significantly increased risk of cancers of the upper aerodigestive tract with an OR of 4.80 (95% CI = 2.39–9.67) (Table 2). After adjustment by age, sex, ethnicity, and smoking status, the relationship was still evident with an OR of 4.84 (95% CI = 2.39–9.81). When we categorized the subjects by the quartile distribution of breaks per cell as in the control subjects, we found a significant gradient of increasing risk of cancer of the upper aerodigestive tract with increasing numbers of induced breaks per cell. The adjusted ORs for subjects in the second, third, and highest quartiles of BPDE-induced breaks relative to the lowest quartile were 4.12 (95% CI = 0.77–22.18), 7.49 (95% CI = 1.49–37.68), and 19.97 (95% CI = 4.24–94.12), respectively; the trend was statistically significant ($P<.001$). The adjusted ORs were 7.57 (95% CI = 2.70–21.22) in younger subjects (<65 years old) compared with 2.82 (95% CI = 1.00–7.97) in older subjects (⩾65 years old), 6.15 (95% CI = 2.73–13.89) for male subjects compared with 2.43 (95% CI = 0.50–11.88) for female subjects, and 3.65 (95% CI = 1.67–7.97) for case subjects with stage I disease compared with 9.25 (95% CI = 2.94–29.10) for case subjects with stage II disease. There was a higher risk in former smokers (OR = 10.94; 95% CI = 3.56–33.63) than in never smokers (OR = 1.53; 95% CI = 0.12–19.12) and even current smokers (OR = 4.73; 95% CI = 1.34–16.78). The median number of the pack-years, the median number of years of smoking, or the median number of cigarettes smoked per day in the control subjects was used as the cutoff point to distinguish light smokers from heavy smokers; we observed a significantly increased OR in light smokers compared with that in heavy smokers.

Nonparametric bootstrap samplings were generated to assess the validity of our model. When 0.60 break per cell was used as a cutoff value for BPDE sensitivity, the risk estimate was 4.94 by the bootstrap method. The 95% CIs by the percentile method and by the biased correction and accelerated method were 2.47–10.48 and 2.45–10.25, respectively. When the BPDE sensitivity cutoff value was set at the 75th percentile value in the control group of the bootstrap sample, the cutoff values ranged from 0.48 to 0.80, with the first quartile, median, and third quartile values found to be 0.58, 0.60, and 0.62, respectively. The bootstrap estimate of the OR for BPDE sensitivity was 5.04 with the 95% CIs being 2.47–10.29 and 2.19–8.88, as determined by the percentile method and by the biased correction and accelerated method, respectively. The parametric bootstrap method was also applied to evaluate the logistic model with multiple covariates. The OR for BPDE sensitivity was 5.19 (95% CI = 2.48–11.53) after adjustment for age, sex, ethnicity, and smoking status in the bootstrap samples. All bootstrap analyses yielded estimates similar to those calculated from the original data, suggesting that the models gave a reasonable fit to the data and

Table 2. Associations between BPDE sensitivity and cancer risk in patients with upper aerodigestive tract cancer and healthy control subjects*

BPDE sensitivity, breaks/cell	No. (%)		Univariate OR (95% CI)	Adjusted OR (95% CI)
	Case subjects	Control subjects		
Overall breaks/cell†				
<0.60	24 (35.8)	59 (72.8)	1.00 (referent)	1.00 (referent)
≥0.60	43 (64.2)	22 (27.2)	4.80 (2.39–9.67)	4.84 (2.39–9.81)
Quartiles of breaks/cell†				
<0.32	2 (3.0)	20 (24.7)	1.00 (referent)	1.00 (referent)
0.32–0.45	8 (11.9)	20 (24.7)	4.00 (0.75–21.22)	4.12 (0.77–22.18)
0.46–0.59	14 (20.9)	19 (23.4)	7.37 (1.47–36.83)	7.49 (1.49–37.68)
≥0.60	43 (64.2)	22 (27.2)	19.55 (4.18–91.32)	19.97 (4.24–94.12)
Age at diagnosis†				
<65 y				
<0.60 break/cell	11 (28.9)	30 (75.0)	1.00 (referent)	1.00 (referent)
≥0.60 break/cell	27 (71.1)	10 (25.0)	7.36 (2.70–20.05)	7.57 (2.70–21.22)
≥65 y				
<0.60 break/cell	13 (44.8)	29 (70.7)	1.00 (referent)	1.00 (referent)
≥0.60 break/cell	16 (55.2)	12 (29.3)	2.97 (1.10–8.04)	2.82 (1.00–7.97)
Sex‡				
Male				
<0.60 break/cell	19 (34.5)	49 (76.6)	1.00 (referent)	1.00 (referent)
≥0.60 break/cell	36 (65.5)	15 (23.4)	6.19 (2.78–13.80)	6.15 (2.73–13.89)
Female				
<0.60 break/cell	5 (41.7)	10 (58.8)	1.00 (referent)	1.00 (referent)
≥0.60 break/cell	7 (58.3)	7 (41.2)	2.00 (0.45–8.96)	2.43 (0.50–11.88)
Ethnicity§				
African-American				
<0.60 break/cell	2 (33.3)	4 (66.7)	1.00 (referent)	1.00 (referent)
≥0.60 break/cell	4 (66.7)	2 (33.3)	4.00 (0.36–44.11)	—‖
White				
<0.60 break/cell	22 (36.1)	55 (73.3)	1.00 (referent)	1.00 (referent)
≥0.60 break/cell	39 (63.9)	20 (26.7)	4.88 (2.35–10.13)	4.88 (2.34–10.19)
Stage†				
I				
<0.60 break/cell	19 (42.2)	59 (72.8)	1.00 (referent)	1.00 (referent)
≥0.60 break/cell	26 (57.8)	22 (27.2)	3.67 (1.70–7.91)	3.65 (1.67–7.97)
II				
<0.60 break/cell	5 (22.7)	59 (72.8)	1.00 (referent)	1.00 (referent)
≥0.60 break/cell	17 (77.3)	22 (27.2)	9.12 (3.00–27.69)	9.25 (2.94–29.10)
Smoking status¶				
Never				
<0.60 break/cell	2 (33.3)	3 (50.0)	1.00 (referent)	1.00 (referent)
≥0.60 break/cell	4 (66.7)	3 (50.0)	2.00 (0.19–20.61)	1.53 (0.12–19.12)
Former#				
<0.60 break/cell	8 (25.8)	36 (73.5)	1.00 (referent)	1.00 (referent)
≥0.60 break/cell	23 (74.2)	13 (26.5)	7.96 (2.86–22.18)	10.94 (3.56–33.63)
Current				
<0.60 break/cell	14 (46.7)	20 (76.9)	1.00 (referent)	1.00 (referent)
≥0.60 break/cell	16 (53.3)	6 (23.1)	3.81 (1.19–12.16)	4.73 (1.34–16.78)
No. of pack-years¶				
<40 pack-years				
<0.60 break/cell	9 (29.0)	26 (76.5)	1.00 (referent)	1.00 (referent)
≥0.60 break/cell	22 (71.0)	8 (23.5)	7.94 (2.62–24.08)	8.57 (2.70–27.25)
≥40 pack-years				
<0.60 break/cell	12 (41.4)	30 (73.2)	1.00 (referent)	1.00 (referent)
≥0.60 break/cell	17 (58.6)	11 (26.8)	3.86 (1.40–10.63)	3.58 (1.24–10.35)
No. of years of smoking¶				
<34 years of smoking				
<0.60 break/cell	6 (26.1)	28 (75.7)	1.00 (referent)	1.00 (referent)
≥0.60 break/cell	17 (73.9)	9 (24.3)	8.81 (2.67–29.15)	9.14 (2.64–31.68)
≥34 years of smoking				
<0.60 break/cell	15 (40.5)	28 (73.7)	1.00 (referent)	1.00 (referent)
≥0.60 break/cell	22 (59.5)	10 (26.3)	4.11 (1.55–10.90)	3.92 (1.45–10.59)
No. of cigarettes¶				
<30 cigarettes				
<0.60 break/cell	13 (30.2)	25 (73.5)	1.00 (referent)	1.00 (referent)
≥0.60 break/cell	30 (69.8)	9 (26.5)	6.41 (2.35–17.46)	6.71 (2.41–18.69)
≥30 cigarettes				
<0.60 break/cell	8 (47.1)	31 (75.6)	1.00 (referent)	1.00 (referent)
≥0.60 break/cell	9 (52.9)	10 (24.4)	3.49 (1.06–11.46)	3.35 (1.00–11.53)

*BPDE = benzo[*a*]pyrene diol epoxide; OR = odds ratio; CI = confidence interval.

†Adjusted by age, sex, ethnicity, and smoking status.

‡Adjusted by age, ethnicity, and smoking status.

§Adjusted by age, sex, and smoking status.

‖Data cells are too small to compute adjusted OR.

¶Adjusted by age, sex, and ethnicity.

#Former smoker is defined as a smoker who had not smoked for at least 1 year.

the robustness of the BPDE sensitivity cutoff values.

The mean number of BPDE-induced breaks per cell by host characteristics is summarized in Table 3. There was no significant association between the mean number of breaks per cell and age, sex, ethnicity, smoking status, and the number of pack-years. There was also no significant difference in the mean number ± SD of BPDE-induced breaks per cell for case subjects with stage II disease (0.87 ± 0.41) compared with those with stage I disease (0.72 ± 0.36). The mean number ± SD of BPDE-induced breaks per cell was 0.66 ± 0.32 for case subjects with surgical treatment only, 0.77 ± 0.37 for case subjects with radiotherapy only, and 1.04 ± 0.53 for case subjects who had received combined modalities. However, only six case subjects had received combined modality treatment. There were no significant differences in the distribution of BPDE sensitivity by prior treatment modality (*P*>.05).

Data for bleomycin and BPDE sensitivity were available for 61 case subjects and 77 control subjects (Table 4). Using subjects who were not sensitive to either mutagen as the referent group, we found that the risks were 4.89 (95% CI = 1.61–14.84) for subjects exhibiting BPDE sensitivity, 7.19 (95% CI = 2.29–22.57) for subjects showing bleomycin sensitivity alone, and 19.15 (95% CI = 6.38–57.49) for subjects with sensitivity to both mutagens. Of the 56 subjects who did not express sensitivity to either mutagen, only nine (16.1%) were case subjects, whereas 47 (83.9%) were control subjects. Of the 36 subjects who were sensitive to both bleomycin and BPDE, 28 (77.8%) were case subjects and eight (22.2%) were control subjects. When values for BPDE and bleomycin sensitivities were incorporated into the logistic model, both coefficients were significant, suggesting that they are independent susceptibility markers and the combined effect had higher predictivity than either one of the markers alone. The Pearson correlation test showed that bleomycin sensitivity and BPDE sensitivity were significantly correlated in control subjects with a correlation coefficient *r* of .46 (*P*<.001). Among case subjects, the correlation coefficient *r* was .24 (*P* = .06).

Table 4. Association of bleomycin sensitivity, BPDE sensitivity, and the risk of upper aerodigestive tract cancer*,†

	BPDE sensitivity	
Bleomycin sensitivity	Not sensitive, <0.6 break/cell	Sensitive, ⩾0.6 break/cell
Not sensitive, <0.6 break/cell		
Univariate OR (CI)	1.00 (referent)	4.82 (1.70–13.70)
Adjusted OR (CI)‡	1.00 (referent)	4.89 (1.61–14.84)
Case subjects/control subjects	9/47	12/13
Sensitive, ⩾0.6 break/cell		
Univariate OR (CI)	6.96 (2.27–21.35)	18.28 (6.40–52.16)
Adjusted OR (CI)‡	7.19 (2.29–22.57)	19.15 (6.38–57.49)
Case subjects/control subjects	12/9	28/8

*BPDE = benzo[*a*]pyrene diol epoxide; OR = odds ratio; CI = 95% confidence interval.
†Data were available for 61 case subjects and 77 control subjects.
‡Adjusted by age, sex, ethnicity, and smoking status.

Table 3. Mean number of BPDE-induced breaks per cell by host characteristics in patients with upper aerodigestive tract cancer and healthy control subjects*

	Case subjects			Control subjects		
Characteristic	No.	Mean breaks per cell (SD)	Two-sided *P*†	No.	Mean breaks per cell (SD)	Two-sided *P*†
Age, y						
<65	38	0.83 (0.44)		40	0.51 (0.29)	
⩾65	29	0.69 (0.27)	.15	41	0.47 (0.21)	.50
Sex						
Male	55	0.74 (0.29)		64	0.46 (0.21)	
Female	12	0.91 (0.65)	.17	17	0.58 (0.35)	.07
Ethnicity						
White	61	0.77 (0.38)		75	0.48 (0.25)	
African-American	6	0.78 (0.43)	.97	6	0.59 (0.34)	.32
Smoking status						
Never	6	0.93 (0.42)		6	0.66 (0.46)	
Former	31	0.74 (0.24)	.12	49	0.47 (0.22)	.10
Current	30	0.77 (0.48)	.45	26	0.48 (0.24)	.19
No. of pack-years‡						
Never smokers	6	0.93 (0.42)		6	0.66 (0.46)	
Light smokers	31	0.85 (0.43)	.66	34	0.45 (0.26)	.13
Heavy smokers	29	0.67 (0.28)	.07	41	0.49 (0.20)	.13

*BPDE = benzo[*a*]pyrene diol epoxide; SD = standard deviation.
†*P* values are based on Student's *t* test.
‡Information was missing from one case subject.

Discussion

We have previously reported that bleomycin-induced mutagen sensitivity was a significant predictor of risk of recurrence of cancer in the overall patient population from which this subgroup is derived *(17)*. We have also shown that there were no significant differences in the distribution of mutagen sensitivity by sex, alcohol use, or dietary consumption of the micronutrients, e.g., α-carotene, β-carotene, lutein, lycopene, or vitamin C *(16)*. As reported previously, there was a tendency (*P* = .05) for case subjects with stage II disease to exhibit a higher mean break per cell of 0.99 compared with 0.90 for case subjects with stage I disease. Case subjects who were surgically treated had a significantly lower mean break per cell than comparable case subjects treated with only radiotherapy. Because of the potential confounding influence of prior treatment on the cytogenetic analysis, we previously categorized case subjects by duration since treatment, dichotomizing at 6 months after the time of receiving treatment. There were no significant differences in the break-per-cell score for case subjects assessed more than 6 months after completion of radiotherapy (mean value = 0.99) compared with those who had been treated more recently (mean value = 0.92). There was also no correlation between the break-per-cell score and months since radiotherapy *(16)*.

This chemoprevention trial included only those case subjects who had early stage cancer and those case subjects who had been previously treated with surgery and/or radiotherapy. Our published retrospective analysis of a different series of 298 newly diagnosed case subjects with previously untreated cancers of the upper aerodigestive tract of all stages at The University of Texas M. D. Anderson Cancer Center *(12)* documented a mean break-per-cell value of 0.98, identical to the mean value in the present series of case subjects with early stage disease, all of whom had been previously treated. Thus, we do not believe that treatment, *per se,* shifts the sensitivity profiles.

The results of this report suggest that case subjects with cancer of the upper aerodigestive tract are significantly more likely to express sensitivity to bleomycin and to BPDE than control subjects. They also support our contention that mutagen sensitivity can be used as a marker in gauging susceptibility to cancer. With regard to the underlying mechanism of the mutagen sensitivity assay, Hsu et al. *(23)* have suggested that it indirectly measures the effectiveness of one or more DNA repair mechanisms. The observations that follow support this hypothesis. First, the relationship between chromosome instability syndromes and cancer proneness is well established. Specifically, the rates of spontaneous as well as bleomycin-induced, γ-irradiation-induced, and UV light-induced chromosome breakage are highest in patients with chromosome instability syndromes such as Bloom syndrome, Fanconi's anemia, ataxia-telangiectasia, Werner's syndrome, and xeroderma pigmentosum. These patients also have defective DNA repair systems *(24–29).* Furthermore, patients with ataxia-telangiectasia, who are extremely sensitive to the clastogenic (mutagenic and carcinogenic) effects of x-irradiation and bleomycin, differ from normal people in the speed with which aberrations induced by these agents are repaired but not in the number of aberrations produced *(30,31).* There is also recently acquired evidence of a correlation between the cellular DNA repair capacity measured by the host cell reactivation assay and the frequency of mutagen-induced *in vitro* chromosome breaks *(32).*

Pandita and Hittelman *(33),* however, observed increased levels of initial chromosome damage (detected by premature chromosome condensation), a reduced activity of the fast-repair component, and relatively high residual chromosome damage in lymphoblastoid cell lines established from patients with multiple primary head and neck tumors and high sensitivity to bleomycin *in vitro* compared with the findings in healthy control subjects. As a result, they hypothesized that mutagen sensitivity involves an inherent chromatin alteration that permits the more efficient translation of DNA damage into chromosome damage after exposure to a mutagen.

On the basis of these collective findings, we hypothesize that the mutagen sensitivity assay detects the composite outcomes of multiple processes. The strong and consistent association between mutagen sensitivity and the risk of cancer of the upper aerodigestive tract indicates that the assay measures a potentially informative aspect of susceptibility to damage or of the repair capacity in those tissues. One may speculate that the cancer patients simply have more chromosome breaks *in vivo* and, thus, may appear to exhibit more breaks after the BPDE (or bleomycin) treatment. We and others *(9)* have extensively studied the incidence of baseline ''spontaneous'' breaks *in vitro.* The frequencies of such spontaneous chromatid breaks are extremely low (±0.02 break per cell) both in normal subjects and in case patients. Thus, we do not routinely report them separately.

Various carcinogens have been implicated in the development of cancer of the upper aerodigestive tract. Prospective and retrospective studies worldwide have consistently shown linear dose–response effects of tobacco exposure on the risk of oral and laryngeal cancers. Blot et al. *(34)* have estimated that tobacco smoking and alcohol consumption together cause approximately three fourths of all oral and pharyngeal cancers in the United States. Because different carcinogens and mutagens act on cells through different molecular mechanisms, these agents may in turn activate different repair pathways. In addition, a person who is sensitive to one mutagen may be resistant to another. For example, we have found that case subjects with skin cancer are sensitive to 4-NQO (4-nitroquinoline *N*-oxide), a UV light-mimetic agent, but they are not necessarily sensitive to bleomycin *(14).*

Our data showed that sensitivity to BPDE, a metabolic product of benzo[*a*]-pyrene, was a significant risk factor for cancer of the upper aerodigestive tract. We also found that BPDE sensitivity, like bleomycin sensitivity, was not affected by the extent of exposure to cigarette smoking. We believe, therefore, that this is a constitutional phenotype. We observed that, similar to bleomycin sensitivity, the risk by BPDE sensitivity was even higher for case subjects who were of a younger age at diagnosis and who were former smokers. The theory is that exposed individuals with sensitive phenotypes might develop cancer earlier and following less carcinogen exposure. Genetic differences in risk tend to be smaller at high doses of carcinogens, when the effect of the carcinogen may overpower any genetic predisposition. Among our current smokers, both case subjects and control subjects generally were heavy smokers, so the risk factor of smoking might significantly outweigh a lesser risk factor such as sensitive phenotype. Results for bleomycin sensitivity and BPDE sensitivity were significantly correlated in control subjects but less so among case subjects. Multiple genetic defects might modulate the BPDE sensitivity assay in case subjects. The sensitivity of both assays appears to be similar (66% for the bleomycin assay and 64.2% for the BPDE assay). The specificity of the bleomycin assay was 77.9% compared with 72.8% for the BPDE assay. We also noted that people who were sensitive to both bleomycin and BPDE were at a 19.2-fold increased risk of cancer of the upper aerodigestive tract. By using both mutagen sensitivity assays in parallel, we may be able to improve our ability to define high-risk populations. This assay requires validation in prospective studies.

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Notes

[1]*Editor's note:* CCOP was established by the National Cancer Institute (NCI) in 1983. Through this program, community physicians work with scientists conducting NCI-supported clinical trials.

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Potentiation of Lonidamine and Diazepam, Two Agents Acting on Mitochondria, in Human Glioblastoma Treatment

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Background: **Cellular metabolism in glioblastoma multiforme, the most common primary brain tumor in humans, is characterized by a high rate of aerobic glycolysis that is dependent on mitochondria-bound hexokinase. Moreover, high levels of glucose utilization and tumor aggressiveness in glioblastoma are associated with a high density of mitochondrial benzodiazepine receptors. We sought to inhibit glioblastoma metabolism by simultaneously inhibiting hexokinase with lonidamine and binding benzodiazepine receptors with diazepam.** ***Methods:*** **Cellular glioblastoma metabolism in five glioblastoma cell lines was assessed *in vitro* by measuring cell proliferation (by use of a tetrazolium-based colorimetric assay, measurement of DNA synthesis, and assessment of cell cycle distribution), by measuring membrane fluidity (by fluorescence polarization measurement of cells stained with a fluorescent probe), and by measuring changes in intracellular pH. Immunodeficient nude mice bearing subcutaneous xenografts of human glioblastoma cells were used to assess the antitumor activities of lonidamine and diazepam; the mice were treated twice daily with lonidamine (total daily dose of 160 mg/kg body weight) and/or diazepam (total daily dose of 1 mg/kg body weight) for 10 consecutive days.** ***Results:*** **When used in combination, the two drugs had a stronger effect on glioblastoma cell proliferation and metabolism *in vitro* than did either agent used alone. *In vivo,* the combination of lonidamine and diazepam was significantly more effective in reducing glioblastoma tumor growth than either drug alone (two-sided $P<.01$, Mann–Whitney U test, comparing growth of treated tumors with that of untreated tumors); this tumor growth retardation was maintained as long as treatment was given.** ***Conclusion:*** **The combination of lonidamine and diazepam—drugs that target two distinct mitochondrial sites involved in cellular energy metabolism—potentiates the effects of the individual drugs and may prove useful in the treatment of human glioblastomas. [J Natl Cancer Inst 1998;90:1400–6]**

Glioblastoma multiforme, the most common primary brain tumor in humans is almost always fatal *(1,2).* The metabolism of gliomas, like that of many tumors, is characterized by a high rate of aerobic glycolysis *(3–5).*

Lonidamine affects energy metabolism by inhibiting mitochondria-bound hexokinase *(6–8)* and the electron transport chain *(9).* Lonidamine alters the cell's plasma and mitochondrial membranes, resulting in inhibition of cellular respiration and depletion of cellular adenosine triphosphate (ATP) *(9);* impedes DNA repair *(10);* and induces cellular acidification *(11).* It has been shown that modification of the intracellular pH (pHi) markedly alters the subcellular distribution of hexokinase in glioma cells *(12).* Treatment of xenografted human gliomas with lonidamine slows the tumor growth rate in correlation with their mitochondria-bound hexokinase activity *(5).*

Hexokinase is bound to porins, channel-forming proteins that are located on the outer mitochondrial membrane *(13)* and that form a complex containing the mitochondrial benzodiazepine receptor (mBzR) and ATP-adenosine diphosphate (ADP) translocase *(14).* The mBzRs are involved in the regulation of growth and differentiation of tumors *(15,16)* and recently have been implicated in an antioxidant pathway that modulates apoptosis *(17).* Increased density of mBzR in glioblastomas was associated with glucose utilization and aggressiveness *(18).*

We used diazepam as a reference agent that interacts with mBzRs and has affinity constants in the nanomolar range *(19).* We studied the effects of diazepam in combination with lonidamine, which alters the metabolism of the glial tumors, on glioblastoma multiforme *in vitro* (on cell proliferation, cell cycling, membrane fluidity, intracellular pH [pHi], and apoptosis) and *in vivo* (on the growth of human glioma transplanted into nude mice).

Materials and Methods

Cell Culture

Five human glioma cell lines were used: SNB-56, SNB-78, and SNB-19 *(20),* TG-8-OZ *(5,21),* and U251 (from Dr. P. A. Steck, The University of Texas M. D. Anderson Cancer Center, Houston). All of the cells were cultured in Dulbecco's modified Eagle medium (DMEM; Sigma Chemical Co., St. Louis, MO) containing 25 m*M* glucose and supplemented with 10% fetal calf serum (Dutscher, Brumath, France), 2 m*M* L-glutamine, penicillin (100 U/mL), streptomycin (50 μg/mL), and epidermal growth factor (Sigma Chemical Co.) (10 ng/mL). Cells were maintained at 37 °C in a humidified 5% CO_2–95% air incubator. For experiments, cells were collected from subconfluent monolayers in a solution of trypsin (0.5 mg/mL) and EDTA (0.2 mg/mL) in phosphate-buffered saline (PBS; Sigma Chemical Co.). Cell viability, estimated by trypan blue exclusion, was greater than 95% before each experiment.

Cell Proliferation Assay

Growth inhibition of the five cell lines by lonidamine and diazepam, alone or in combination, was studied by using the microculture 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.) assay. The amount of tetrazolium dye reduced to formazan is proportional to the number of viable cells. Briefly, 3×10^3 cells were plated per well in 96-well polystyrene plates (ATGC, Noisy-le-Grand, France) in culture medium and allowed to adhere overnight. The medium was then replaced with fresh medium containing increasing concentrations of lonidamine (Doridamine; supplied by F. Angelini S.p.a., Rome, Italy) from 25 to 600 μ*M,* diazepam (Valium®; Roche, Neuilly-s/Seine, France) from 18 to 350 μ*M,* or both agents. Diazepam and lonidamine were further diluted in DMEM from freshly prepared stock solutions of ethanol and *N*-methyl D-glucamine (Sigma Chemical Co.), respectively, and then were added directly to the medium to obtain the appropriate final concentration in a total volume of 200 μL. After a 96-hour incubation, the medium was

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See ''Notes'' following ''References.''

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removed, and the plates were washed with PBS. Each well received 100 μL of phenol red-free DMEM (Sigma Chemical Co.) supplemented with 0.1 volume of an MTT solution (5 mg/mL in PBS), and the plates were incubated at 37 °C for 4 hours. The MTT-containing medium was then aspirated, 100 μL of dimethyl sulfoxide (DMSO; Sigma Chemical Co.) was added to each well, and the plates were gently agitated until the MTT formazan had dissolved. Growth inhibition was quantified as the decrease in the amount of the tetrazolium dye reduced to formazan by the treated cells versus control cells; formazan was measured as absorbance at 550 nm in an LP500 spectrophotometer (J Bio, Les Ulis, France). Treated (six wells/concentration) and control (12 wells/plate) cells always contained less than 0.1% *N*-methyl D-glucamine and 0.5% ethanol, which do not inhibit cell proliferation. Absorbance of control culture was taken as 100%, and absorbance of the other cultures was expressed as a percent of the control. Each assay was repeated twice.

[^{3}H]Thymidine Uptake Assay

[^{3}H]Thymidine uptake was used to assess the effects of the drugs on DNA synthesis in short-term assays. Culture and treatment conditions were as described for the MTT assay. After 2 hours of drug exposure, each well received 1 μCi (5 μL) of [^{3}H]thymidine (20 Ci/mmol; Du Pont NEN, Les Ulis, France), and the incubation was continued for 4 hours. The cells were then harvested on glass fiber filters (Wallac, Turku, Finland) by a Micro Cell Harvester (Skatron, OSI, Paris, France) and washed. The dried filters were placed into a sample bag containing 10 mL of OptiScint ''HiSafe'' fluid, and radioactivity was measured in a 1205 Betaplate liquid scintillation counter (Wallac). Six identical samples were used for treatment, and 12 identical samples were used for control points. The mean counts per minute were calculated, control radioactivity was taken as 100%, and the experimental values are expressed as a percent of the control.

Cell Cycle Determination

Cells were plated in 75-cm^2 plastic culture flasks and allowed to adhere for 24 hours. Medium was then replaced with fresh medium containing lonidamine and/or diazepam for 48 hours. The cell cycle was assessed according to Demarcq et al. *(22).* Briefly, 30 μ*M* bromodeoxyuridine (BrdUrd, Sigma Chemical Co.) was added for 15 minutes. The cells were then washed three times with PBS and collected after trypsinization. After washing, the cells were suspended in 0.5 mL of PBS and fixed by slowly adding 1.5 mL of ice-cold ethanol. After overnight incubation at 4 °C, the cells were permeabilized, incubated first with a rat anti-BrdUrd antibody (Seralab, Sigma Chemical Co.) diluted 1:25 in buffer and then with fluorescein isothiocyanate-conjugated goat anti-rat antibody (Cliniscience, Paris, France) diluted 1:50. Cells were incubated with propidium iodide in PBS (20 μg/mL; Sigma Chemical Co.) and then subjected to flow cytometry (Becton Dickinson, San Jose CA). Data were analyzed by using a LYSIS II computer program on 10^4 cells and expressed as histograms. Percentages of cells present in areas corresponding to each phase of the cell cycle were calculated.

Membrane Fluidity Measurements and Fluorescence Instrumentation

The fluorescent probe 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH; Molecular Probes, Eugene, OR) was used to investigate membrane fluidity. Because of its positive charge, which provides a plasma membrane surface anchor, TMA-DPH, a cationic derivative of DPH, has greater specificity of localization than DPH in the plasma membrane *(23).*

The SNB-19 human glioma cells, grown in 75-cm^2 flasks, were treated with 100 μ*M* lonidamine and/or 88 μ*M* diazepam or with their respective vehicles (0.2% *N*-methyl D-glucamine and 0.5% ethanol) for 24 or 48 hours. After the end of the incubation period, attached cells were washed twice with PBS, trypsinized, and resuspended in PBS at a concentration of 10^6 cells/mL. 1.5 μL TMA-DPH (1 m*M* dissolved in tetrahydrofuran) was then added to 3 mL of the cell suspension (final concentration, 1 μ*M*) and incubated in the dark at 25 °C for 10 minutes.

Steady-state fluorescence anisotropy and fluorescence intensity were measured with a continuous excitation instrument (Fluofluidimètre, Régulest, Villers-lès-Nancy, France) with a thermostatically controlled sample holder at 25 °C. The excitation wavelength was 365 nm, and emission was read at 425 nm *(24).* Corrections for light scattering (probe-free cell suspensions) were performed routinely. The steady-state fluorescence anisotropy ($\langle r \rangle$) was obtained with the following equation:

$$\langle r \rangle = [(I_{//} - I_{b//}) - G(I_{\perp} - I_{b})]/[(I_{//} - I_{b//}) - 2G(I_{\perp} - I_{b})],$$

where I// and I⊥ are the fluorescence intensity measured on the analyzing polarizer parallel or perpendicular to the polarized excitation beam, respectively; *G* is an instrumental correction factor used to correct the unequal transmission of differently polarized light; and *b* is the blank (unlabeled cells). Membrane fluidity is expressed as $\langle r \rangle$, which is inversely related to fluidity.

Fluorescence lifetimes were determined with an Aminco-SLM 48000-S apparatus (SLM Instruments, Urbana, IL), which works on the principle of phase and frequency modulation *(24).* A wide range of frequencies between 15 and 150 MHz was used for the fluorescent lifetime measurements of TMA-DPH; all lifetime measurements were obtained by using diphenylanthracene (Sigma Chemical Co.) in the reference cell. The data were analyzed by a discrete exponential components model. A nonlinear least-square routine was used, and the reduced c^2 was taken as the goodness criterion of the fit.

DNA Fragmentation Analysis

DNA fragmentation was analyzed after conventional 1.2% agarose gel electrophoresis. Cultured SNB-19 cells (2×10^7 cells) were treated with lonidamine and/or diazepam for 24 and 72 hours. At the end of the incubation period, all floating and attached cells were collected and washed three times in serum-free medium. DNA was extracted and analyzed by agarose gel electrophoresis as described *(25).*

Detection of Apoptosis by Flow Cytometry

Lonidamine- and/or diazepam-induced apoptosis was detected by flow cytometric analysis of permeabilized propidium iodide-stained cells as described by Shih and Stutman *(26).* Cell cycle analysis was performed, and G_0/G_1 phase was determined. Search for a sub-G_1 population characteristic of cells undergoing apoptosis was done.

Membrane Preparation and [^{3}H]Diazepam-Binding Study

Membranes were prepared from xenografted human glioma TG-8-OZ and from human glioma cells in culture. Briefly, 2×10^8 glioma cells were used to prepare membranes for receptor-binding studies. Each extract of tumors or glioma cells was homogenized in 20 volumes of ice-cold 50 m*M* Tris–HCl (pH 7.4) in an Ultra Turax homogenizer at maximum speed for 10 seconds. The resulting homogenate was centrifuged at 1500*g* for 10 minutes at 4 °C. The pellets, containing cell debris and nuclei, were discarded and the supernatants were centrifuged at 30 000*g* for 20 minutes at 4 °C. The pellets were resuspended in 20 volumes of buffer, and the washing was repeated once more. The resulting membrane suspensions were frozen at −80 °C until assayed. Protein was measured with the BCA protein assay (Pierce, Rockford, IL) using bovine serum albumin as the standard. [^{3}H]Diazepam (83 Ci/mmol; Du Pont NEN) binding was assayed as described *(27).* Each sample contained 0.1 mg of membrane protein and [^{3}H]diazepam. The assays were performed in triplicate in 50 m*M* Tris–HCl (pH 7.4) in a final volume of 0.5 mL. Incubations were carried out at 4 °C for 30 minutes, and each sample was transferred by vacuum filtration onto 25-mm Whatman GF-C filters via a Model 1225 Vacuum Manifold (Millipore, Bedford, MA) followed by four 3-mL washes with ice-cold buffer. Nonspecific binding was determined with 10 μ*M* unlabeled diazepam.

Drug Treatment and pHi Determination

The SNB-19 cell line was cultivated in 35-mm tissue culture dishes for 24 hours (Costar, Cambridge, MA). The culture medium then was replaced by buffer (10 mM Hepes, 130 mM NaCl, 20 mM KCl, 1 mM $CaCl_2$, 1 mM KH_2PO_4, 0.5 mM $MgSO_4$, and 5 mM glucose, pH 7.4). After 5 minutes at 37 °C, the cells were treated with various doses of lonidamine (dissolved in 2.3% *N*-methyl D-glucamine), diazepam, or both drugs for various periods of time at 37 °C. *N*-methyl D-glucamine did not exceed 0.5%. Cells were then loaded with semi-naphthorhodafluor-1-acetoxymethylester (SNARF-1-AM; Molecular Probes) for 15 minutes at 37 °C. SNARF-1-AM, a rhodamine derivative that generates two pH-sensitive bands in its fluorescence emission spectrum, enables pHi determination by microspectrofluorometry under defined staining conditions *(28).* SNARF-1-staining of SNB-19 cells was obtained as described *(12).* The calibration curve was performed as described *(28).* The pH equilibration technique of Thomas et al. *(29),* using nigericin (1 μg/mL) and valinomycin (5 μ*M*) in a high K^+ buffer (130 m*M*) previously adjusted to a different pH, enabling a rapid equilibration of exter-

nal pH (pHo) and pHi, was used. The fluorescent probe (1 m*M* dissolved in DMSO) was used at a final concentration of 5 μ*M* (0.5% DMSO). Laser microspectrofluorometry of SNARF-1-stained cells was as described *(12)*.

Antitumor Activity Against Subcutaneous Glioma in Nude Mice

Swiss mice (nu/nu, female), weighing 30 g and being 6–8 weeks old, were bred in the animal facilities (Institut Curie, Paris, France) and maintained under specified pathogen-free conditions with artificial lighting (12-hour light/12-hour dark cycle) and fed a regular diet and water *ad libitum.* The care, housing, and handling of the mice were performed in accordance with the recommendations of the French Ethics Committee and under the supervision of authorized investigators.

The TG-8-OZ glioma was established by subcutaneous implantation of human tumor fragments into the scapular area of nude mice under ether anesthesia administered for a short period *(5)*. Tumors appeared after 2 weeks and were allowed to reach a volume of 500 mm^3, at which time they were serially transplanted into other mice (from three to 10 times). The karyotype was monitored to confirm the human origin of the tumor *(21)*. For therapeutic assays, the tumor-bearing mice were randomly divided into groups of ten. The xenografted TG-8-OZ tumors were treated as soon as the mean tumor diameter reached 5 mm (or a tumor volume of approximately 60 mm^3). Mice received diazepam (total daily dose of 1 mg/kg [body weight] in physiological saline), lonidamine (total daily dose of 160 mg/kg [body weight] in intralipid 20%; Kabi Pharmacia, Sweden), or both drugs in two intraperitoneal injections (each containing one half of the total daily dose) for a period of 10 days. The control group received injections of intralipid alone. All mice were weighed twice a week.

Tumor growth was monitered by measuring two perpendicular diameters with a caliper. Tumor volume (*V*) and the relative tumor volume (RTV) were calculated *(30)* as follows:

$$V = a^2 \times b/2,$$

where a is the width (large diameter) and b the length of the tumor in millimeters (small diameter).

$$\mathrm{RTV} = V_x/V_i,$$

where V_x is the mean tumor volume (of 10 mice/group) in cubic millimeters at any given time and V_i is the mean initial tumor volume in cubic millimeters at the beginning of treatment; computation of the RTV allowed the tumor growth to be compared despite initial differences in volume. The response of xenografted TG-8-OZ tumors was also assessed by growth delay, expressed as T – C, which is defined as the difference in days between the median time for the tumors of treated (T) and control (C) animals to reach a volume five times greater than the volume recorded at the time of treatment *(31)*. Mice were killed when the tumor volume reached 2500 mm^3 in the control group.

To evaluate the plasma concentration of diazepam, a radioisotope assay using mice treated as described above was performed. The last injection contained 1 mg/kg (body weight) diazepam and 8.7 μCi of [^{3}H]diazepam (1:1000 dilution). One hour later, the plasma was recovered and the radioactivity in it was measured.

Statistical Analysis

We used the Mann–Whitney *U* test to assess the effects of diazepam on the ability of lonidamine to inhibit cell proliferation and [^{3}H]thymidine uptake, alter pHi, and decrease membrane fluidity. The statistical significance of the differences in the volume for nontreated and treated tumors was also tested by use of the Mann–Whitney *U* test. Tests were two-tailed; the *P* values are two-sided and were considered to be significant if they were less than or equal to .05.

Results

Effects of Lonidamine and Diazepam on Cell Proliferation, [^{3}H]Thymidine Uptake, and Cell Cycle

Inhibition of glioma cell proliferation was measured by using the MTT test 96 hours after the start of incubation in increasing concentrations of lonidamine, diazepam, or both (Table 1). The concentration of lonidamine alone required to produce 50% inhibition of growth (IC_{50}) was 200 μ*M*. Diazepam alone at concentrations of 17.5 to 88 μ*M* did not inhibit cell growth; however, a 66% growth inhibition was obtained at 175 μ*M* diazepam. When the two drugs were combined, for the five cell lines studied, diazepam enhanced lonidamine-induced growth inhibition in a dose-dependent manner. A nonantiproliferative concentration of diazepam (88 μ*M*) was able to lower the IC_{50} value of lonidamine significantly (P<.01 compared with lonidamine used alone), in the range of 1.8–fold to fivefold in these five glioma cell lines.

The effects of lonidamine and/or diazepam on [^{3}H]thymidine uptake were determined in a 6–hour incubation assay, a period chosen to avoid significant reduction of cell number (Table 1). The lonidamine effect on [^{3}H]thymidine uptake was potentiated by diazepam. Lonidamine alone (200 μ*M*) reduced the percentage of incorporation in four cell lines and was ineffective at 100 μ*M.* Diazepam alone (175 μ*M*) decreased the percentage of incorporation (28%–52%) and was ineffective at a lower concentration (88 μ*M*). In a medium containing 100 μ*M* lonidamine and 88 μ*M* diazepam, all five cell lines incorporated significantly less [^{3}H]thymidine (P<.01 compared with each agent used alone) than the lines did in the presence of lonidamine alone.

Table 1. Effects of lonidamine (LND) and diazepam (DZ) used alone or in combination on glioma cell proliferation* and [^{3}H]thymidine uptake†

	Human glioma cell lines‡				
	SNB-19	SNB-56	SNB-78	TG-8-OZ	U251
IC_{50} DZ μ*M*	220 ± 30	170 ± 30	200 ± 28	140 ± 25	196 ± 32
IC_{50} LND μ*M*	200 ± 35	200 ± 30	180 ± 30	170 ± 30	280 ± 50
IC_{50} LND in presence of DZ, μ*M*					
DZ (17.5 μ*M*)	190 ± 40	150 ± 30	100 ± 30	130 ± 40	280 ± 50
DZ (44 μ*M*)	100 ± 40	120 ± 30	50 ± 10§	120 ± 30§	180 ± 50
DZ (88 μ*M*)	50 ± 15§	60 ± 6§	35 ± 5§	90 ± 25§	150 ± 40§
DZ (175 μ*M*)	20 ± 8	—	20 ± 7	—	80 ± 7
[^{3}H]Thymidine uptake, % of control					
LND (0 μ*M*)-DZ (175 μ*M*)	28 ± 5	35 ± 5	32 ± 2	45 ± 6	52 ± 5
LND (0 μ*M*)-DZ (88 μ*M*)	81 ± 7	95 ± 6	97 ± 2	88 ± 6	90 ± 5
LND (200 μ*M*)-DZ (0 μ*M*)	89 ± 6	65 ± 4	65 ± 4	43 ± 7	42 ± 5
LND (200 μ*M*)-DZ (88 μ*M*)	38 ± 7‖	30 ± 6‖	43 ± 4‖	39 ± 6	19 ± 7
LND (100 μ*M*)-DZ (0 μ*M*)	100 ± 3	93 ± 3	95 ± 2	90 ± 4	87 ± 3
LND (100 μ*M*)-DZ (88 μ*M*)	54 ± 6‖	48 ± 5‖	60 ± 6‖	53 ± 6‖	27 ± 8‖

*Effects of lonidamine and diazepam, used alone or in combination, on the proliferation of five glioma cell lines were evaluated with the MTT test. The IC_{50} value (μ*M*) of lonidamine alone or in combination with various concentrations of DZ was determined after 96 hours of culture as indicated.

†Lonidamine and/or diazepam treatment on five glioma cell lines was as described for MTT assay except that, after 2 hours of incubation, 1 μCi of [^{3}H]thymidine was added to each well and the incubation was allowed to proceed for 4 hours. Radioactivity was then counted and the values are expressed as percentages of control values, the control corresponding to the incorporation of [^{3}H]thymidine in untreated cells.

‡Each value represents the mean ± standard error of three experiments.

§P<.01; IC_{50} values of lonidamine used alone were compared to the IC_{50} values of lonidamine obtained in presence of diazepam. *P* values are two-sided and were significant when P<.05 compared to lonidamine used alone.

‖P<.01 compared with each agent used alone. *P* values are two-sided and are significant when P<.05 compared to each agent used alone.

Cycling of SNB-19 cells treated for 48 h with various concentrations of lonidamine and diazepam, alone or in combination, was analyzed. When 100 or 200 μ*M* lonidamine was used alone, the S-phase component of the cell cycle decreased slightly (3% or 9%, respectively; Table 2). Diazepam (88 or 175 μ*M*) alone also decreased the S-phase fraction (4% or 25%, respectively). The G_0/G_1-phase arrest, observed with the highest diazepam concentration, was comparable to that obtained with 100 μ*M* lonidamine and 88 μ*M* diazepam in combination (approximately 30% higher than control). This combination diminished the S-phase fraction by half. The G_2 + M-phase fraction remained stable regardless of the treatment given.

Table 2. Effect of lonidamine (LND) and diazepam (DZ) on the cell cycle in SNB-19 cells*

LND, μ*M*	DZ, μ*M*	% of cells $G_0 + G_1$ phases	S phase	G_2 + M phases
0	0	51	32	15
100	0	49	29	16
200	0	58	23	16
0	88	57	28	13
0	175	73	7	19
100	88	72	15	11
200	88	66	11	13

*Exponentially growing cultures of SNB-19 cells were treated for 48 hours with the drugs at the concentrations indicated. The percentages of the total cell population in each phase of the cell cycle were determined by flow cytometry after pulse-labeling with bromodeoxyuridine (BrdUrd) and BrdUrd/DNA double staining. Each value represents the mean of two experiments performed in triplicate (standard deviation represents less than 10% of the mean).

Effect of Drug Treatment on Apoptosis

Agarose gel electrophoresis of DNA extracted from SNB-19 cells that had been treated for 24 or 72 h with lonidamine and/or diazepam showed no DNA fragmentation characteristic of apoptosis. Population of cells with DNA content below that of G_0/G_1 phase is considered as apoptotic, due to chromatin condensation *(26).*

Effect of Drug Treatments on pHi

Drug-induced pHi modifications were monitored with the specific pH probe SNARF-1-AM by measuring the fluorescence intensity (FI) of two bands (635 and 592 nm) of its emission spectrum and calculating of the ratio: $R = FI_{635}/FI_{592}$. The calibration curve giving an estimate of pHi values was performed. The results of *R* values are expressed as the mean (± standard deviation) of individual *R* values obtained from the analysis of 20 cells of two different determinations. The mean *R* values, increased from .48 ± .020 at pHo 6 to .77 ± .040 at pHo 8. pHi changes were followed in SNB-19 cells that had been treated with lonidamine and/or diazepam at various doses after 30 or 120 minutes of incubation. Lonidamine (100 μ*M)* used alone produced a time-dependent acidification (pHi 6.85 and 6.5 after 30 minutes and 120 minutes of treatment, respectively), whereas diazepam alone did not significantly affect pHi, even at high concentrations. A mean *R* value of .61 ± .036 (pHi 7.2) was obtained for control cells, but combined 100 μ*M* lonidamine and 88 μ*M* diazepam treatment for 30 and 120 minutes led to decreased *R* values: .52 ± .017 (pHi 6.5) and .48 ± .023 (pHi 6.15), respectively ($P<.01$, compared with each agent used alone). When cells were treated at a high diazepam concentration (175 μ*M*) in combination with 100 μ*M* lonidamine for 30 or 120 minutes, a significant intracellular acidification occurred (pHi 6.25 and 6.0, respectively; $P<.01$, compared with each agent used alone). However, these changes in pHi values must be interpreted with caution because, for those cells with a pHi less than or equal to 6.5, SNARF-1 has a pK_a of about 7.4, which makes it most useful for measuring pH changes in the pH 6.4 to pH 8.4 range, where dR/dpH is optimal *(28).*

Membrane Fluidity Studies

To determine whether the drugs affected plasma membrane fluidity, fluorescence polarization was performed with the TMA-DPH probe. The results of fluidity are expressed as the mean (± standard error) of duplicate anisotropy values (<*r*>) of three different determinations. Membrane fluidity is expressed as <*r*>, which is inversely related to fluidity. Cells grown in medium containing both 100 μ*M* lonidamine and 88 μ*M* diazepam for 24 and 48 hours had significantly lower plasma membrane fluidity (<*r*> values, .272 ± .0012 and .267 ± .0015, respectively; $P<.05$), when compared with the control (<*r*> values, .261 ± .0013 and .259 ± .0018, respectively) and to the cells treated with lonidamine (<*r*> values, .265 ± .0018 and .262 ± .0014, respectively) and diazepam (<*r*> values, .267 ± .0020 and .260 ± .0021, respectively) used as single agents. Lonidamine (200 μ*M*) induced a stronger decrease in membrane fluidity; <*r*> values of .275 ± .0015 and .278 ± .0019 were recorded after 24 and 48 hours of treatment, respectively. Treatment with lonidamine and/or diazepam failed to influence the fluorescence lifetime of TMA-DPH, which ranged from 9.3 to 9.7 nanoseconds.

[^{3}H]Diazepam-Binding Studies

In a typical experiment with mitochondrial membranes (Fig. 1, A), the specific binding of [^{3}H]diazepam was saturable, and nonspecific binding increased linearly, although only slightly, with increasing concentrations of the labeled ligand. Scatchard analysis showed a single class of sites with a dissociation constant (K_d) of 12 ± 4 n*M* (mean ± standard deviation) and a B_{max} (maximal amount of ligand bound) of 693 ± 203 fmol/mg of protein (Fig. 1, B). The Hill coefficient was 1.1, indicating the homogeneity and noncooperative nature of these binding sites. Radiolabeled diazepam binding was not inhibited by 10 μ*M* clonazepam (the central-selective benzodiazepine), indicating that all benzodiazepine sites on mitochondrial membranes are of the peripheral type (data not shown). The five cell lines exhibited similar binding characteristics with K_d values ranging from 15 to 18 n*M* and B_{max} values from 350 fmol/mg of protein to 610 fmol/mg (data not shown).

Assessment of Glioma Growth Inhibition by Lonidamine and/or Diazepam in Nude Mice

Tolerance and efficacy of lonidamine in mice were as determined *(5).* Lonidamine and diazepam were administered at doses of 160 mg/kg (body weight) per day, and 1 mg/kg (body weight) per day, respectively. The doses were injected intraperitoneally, divided into two daily injections given for a period of 10 days. The growth rates of the xenografted TG-8-OZ glioma under lonidamine and/or diazepam therapy are shown in Fig. 2. The optimal growth inhibition was 78% (Mann–Whitney *U* test, two-sided, $P<.01$) 7 days

after initiation of the combined treatment associating lonidamine with diazepam (1 mg/kg, [body weight]), and no significant growth inhibition was observed with either drug alone versus control. A growth delay of 10 days was found, expressed as T – C. Growth curves demonstrated that this inhibitory effect was maintained as long as the treatment was given (until day 10). As soon as the treatment was stopped, tumors resumed their growth in all mice; growth inhibition was 57% (P<.05) 5 days after the end of the treatment (day 15) and 26% 11 days later (day 21; data not shown). No tumor regression was observed. No loss of body weight was registered, and no mice died during the treatment. In similar conditions, carmustine used as a reference chemotherapeutic agent was ineffective (data not shown).

In mice, intraperitoneal daily administration of diazepam (1 mg/kg, [body weight]) for 10 days resulted in a diazepam concentration in plasma of 240–300 ng/mL (determined 1 hour after [^{3}H]diazepam injection). No tranquilizer side effects of diazepam (asthenia, dizziness, sleepiness, and weight loss) were observed at this dose.

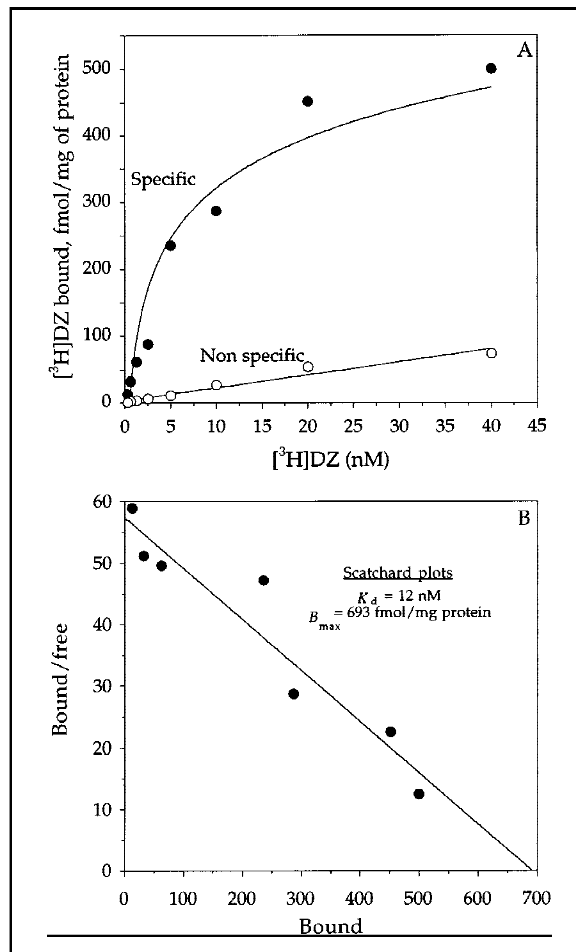

Fig. 1. Saturation and Scatchard plots of [^{3}H]diazepam ([^{3}H]DZ) binding to mitochondrial membranes extracted from xenografted human glioma TG-8-OZ tumor. **A**) Binding assays were performed as described in the ''Materials and Methods'' section. A typical experiment is shown. The specific binding of [^{3}H]diazepam was saturable, and nonspecific binding increased linearly but only slightly with increasing concentrations of the radiolabeled ligand. **B**) Scatchard plot of the binding data using a least-square fit to determine the regression line (r = .96). Scatchard analysis of the specific binding indicated a single class of sites.

Discussion

Our laboratory and others have reported that agents that inhibit glucose metabolism can block the proliferation of malignant glioma cells, both *in vitro* and *in vivo (5,32)*. Lonidamine is a well-tolerated compound that has already undergone phase II and III clinical testing against several human cancers *(33)*. Although lonidamine alone exerts only a weak effect on experimental tumors, it was considered to be a promising drug to be given associated with other agents *(33)*.

The purpose of this study was to attempt to enhance lonidamine efficacy in glioma treatment. Lonidamine efficacy is attributable to its propensity to disrupt plasma and mitochondrial membranes and to reduce glycolysis by a mechanism involving mitochondria-bound hexokinase *(6,7,9,34)*. A relationship between lonidamine efficacy and mitochondria-bound hexokinase activity has been demonstrated *in vivo (5)*.

Like mitochondria-bound hexokinase, mBzRs are located on the outer mitochon-

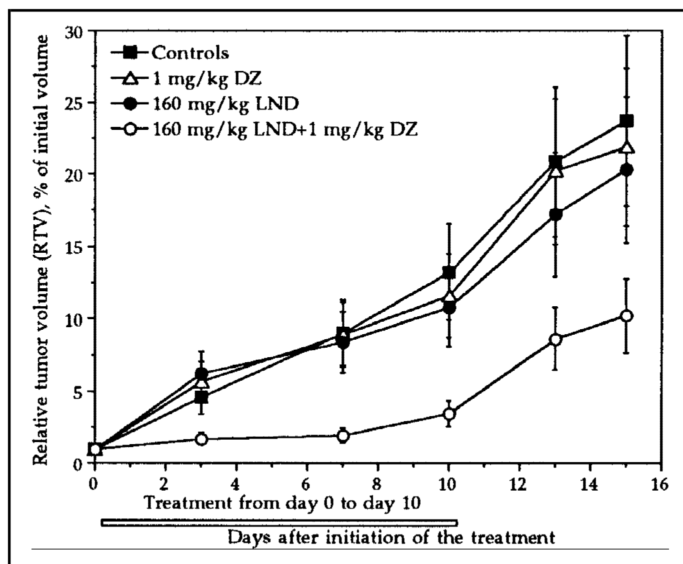

Fig. 2. Antitumor activity of lonidamine (LND) plus diazepam (DZ) treatment on TG-8-OZ human glioblastoma multiforme xenografts. Compounds were administered intraperitoneally twice daily (i.e., each injection containing one half of the total daily dose) for 10 days beginning when the tumor reached a volume of approximately 60 mm^3, as indicated. Each group consisted of 10 mice. The Mann–Whitney U test was used to compare tumor sizes among different treatment groups at day 7 and day 15 after initiation of the treatment. No significant growth inhibition was observed with either drug used alone versus control. Combined therapy significantly inhibited tumor growth: 78% and 57% on day 7 and day 15 after initiation of the treatment, respectively (Mann–Whitney U test, two-sided, P<.01 and P<.05, respectively). A growth delay of 10 days was found, expressed as T – C, defined as the difference in days between the median time for the tumors of treated (T) and control (C) animals to reach a volume five times greater than the volume recorded at the time of treatment. No tranquilizer side effects (asthenia, dizziness, sleepiness, and weight loss) of diazepam at this dose were observed in mice and no mice died during the treatment. Errors bars show the standard deviation.

drial membrane, where they are part of a complex of unknown function containing hexokinase, porins, ATP-ADP translocase, and mBzR *(14).* By using diazepam, an agonist ligand of mBzR, our purpose was to interfere with this mitochondrial energy command center in the hope of increasing the antitumoral effect of mitochondria-bound hexokinase inhibition by lonidamine. This approach was justified by our previous studies showing the critical role of mitochondria-bound hexokinase in the energy metabolism of gliomas *(5),* and this study confirms the high densities of mBzR in these tumors.

The proliferation inhibition induced by the lonidamine and diazepam combination was associated with cell-cycle changes that were not seen when the drugs were used alone. Apoptosis was not induced by the lonidamine and diazepam treatment of the cells for 24 and 72 h. Induction of apoptosis is frequently associated with cell cycle alteration *(35).* Because cells accumulate in the G_0/G_1 phase as a result of the combined treatment, p53-dependent apoptosis may be favored when G_1 phase arrest is not obtained or can not be sufficiently prolonged. Del Bufalo *et al. (36)* recently demonstrated that lonidamine-induced apoptosis was independent of p53 in cells lacking endogenous p53 or overexpressing either wild-type p53 or a dominant-negative p53 mutant. Thus, we suggest that the combination of lonidamine, diazepam, and anticancer agents that act at the G_1 phase of the cell cycle could potentiate the induction of apoptosis and lead to cytotoxicity in a perspective of clinical application and should be tested.

An alternative to death by apoptosis is necrosis, a passive process resulting from altered osmoregulation and/or breaches of membrane integrity. Investigations using the fluorescent probe TMA-DPH demonstrated that treatment with a combination of lonidamine and diazepam decreased membrane fluidity more than treatment with each agent alone. The mBzRs are involved in membrane phospholipid methylation, a process that regulates fluidity *(37).* Lonidamine is a highly hydrophobic molecule that interacts with cellular membranes through its affinity for the inner leaflet of the lipid bilayer *(34).* The reduced fluidity obtained with the combination of lonidamine and diazepam could also contribute to antitumoral efficacy of the combination by altering membrane enzymes, signal transduction, receptors, and channel activities.

Ben Horin et al. *(11)* stated that intracellular acidification induced by lonidamine accounts for some of its cytotoxic effect and its synergy with other therapeutic modalities, such as radiation, hyperthermia, and chemotherapy. We previously reported that imposing a pHi change from pH 6 to pH 8 in SNB-19 cells for 5 min was able to dramatically alter mitochondria-bound hexokinase activity without affecting glycolytic parameters *(12).* Intracellular acidification of cells treated for 2 hours with lonidamine alone confirms earlier observations *(11).* Lowering of the pHi might be in part responsible for lonidamine-induced changes of the energy metabolism of tumor cells. These data have been questionned by Fanciulli et al. *(8)* who demonstrated in intact cells that the inhibition of energy metabolism induced by lonidamine must be primarly ascribed to its effect on mitochondria-bound hexokinase.

The benzodiazepine group of drugs are known to have a pleiotropic spectrum of effects (proliferation inhibition, humoral immune response stimulation, phospholipid methylation, and differentiation induction) *(38).* In glia and human glioma cell cultures, mBzRs regulate the synthesis of neurosteroids, which may directly interfere with gene expression and affect glucose metabolism *(39).*

Our data are consistent with data from other laboratories showing an inhibitory effect of mitochondrial-type benzodiazepines on the proliferation of various cells *(15,40,41).* In human glioma cells, diazepam and mitochondrial-acting ligands (such as PK11195 and Ro5–4864) seem to be more effective in inhibiting DNA synthesis than other benzodiazepines *(40).* Because nanomolar concentrations of diazepam or PK11195 can induce mitoses and cell proliferation *(42),* it has been postulated that mBzRs are involved in the relationship between mitochondrial-acting benzodiazepines and regulation of mitosis *(43).* The association of high levels of mBzR with a high cellular proliferation rate suggests that high mBzR expression is a characteristic feature of malignant cells. High mBzR expression is significantly associated with poor survival of patients independently of histological grade *(18).* Although the mechanisms by which mitochondrial benzodiazepines exert their antiproliferative action remain obscure, they may offer therapeutic potential in glioma *(40).*

Finally, a synergistic antitumor activity of the combined treatment was demonstrated *in vivo* by using glioblastoma TG-8-OZ xenografted into nude mice, a tumor that was found to be insensitive to lonidamine at 250 mg per kg (body weight) per day for 10 days *(5).*

In mice, daily intraperitoneal administration of diazepam (1 mg/kg) for 10 days produced a diazepam concentration in plasma of 240–300 ng/mL. In humans, this plasma concentration can be reached after daily oral administration of 15 mg of diazepam for 10 days; peak plasma drug level occurred at 30–90 minutes and reached values of approximately 300 ng/mL *(44,45).* For its anticonvulsant effect, steady-state diazepam levels that are more than 400–500 ng/mL seem to be required *(46).* The fact that no permanent impairment of cell growth was produced by the combined treatment could have important implications for its potential therapeutic applications. However, treatment with lonidamine and diazepam, combined with chemotherapeutic agents that target other mitochondria-dependent functions that may lead to completing the apoptotic process, might improve the therapy for gliomas.

The potentiation of the antitumor effect of these two drugs, chosen for their respective mitochondrial targets, *in vitro* and *in vivo,* may constitute a new therapeutic approach focusing on the energy metabolism of these highly chemoresistant tumors.

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Notes

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