ABSTRACT

The hypothesis that the frequency distribution of indices of oxidative drug-metabolizing activity is different between patients with bladder cancer (n = 98) and age, sex-matched control subjects (n = 110) has been investigated. Urinary recovery ratios of debrisoquine and R/S ratios of mephenytoin have been measured in an 8-h urine sample after simultaneous administration of debrisoquine (10 mg) and racemic mephenytoin (100 mg). In addition, alcohol consumption, smoking habit, and acetylation phenotype (using 100 mg dapsone as a substrate) have been measured. Patients with bladder cancer were classified on histological criteria as having aggressive (Stage III) (34%) or nonaggressive (Stages I and II) (66%) disease. The median of the frequency distribution of the debrisoquine urinary recovery ratio in patients with aggressive bladder cancer was greater than in control subjects, and only four patients had recovery ratios lower than the mean of the control group. Using logistic regression analysis, efficient debrisoquine metabolism and a synergistic interaction between smoking and ethanol consumption were significant, independent risk factors, while S-mephenytoin hydroxylation and acetylation phenotype were not significant risk factors. In contrast, patients with nonaggressive bladder cancer had a significant, but weaker, association with rapid hydroxylation of S-mephenytoin, which was independent of a significant synergistic interaction between smoking and alcohol consumption. Acetylation phenotype and debrisoquine urinary recovery ratio were not associated with increased risk of nonaggressive cancer. These results are consistent with the concept that oxidative isozymes might be responsible for conversion of environmental agents to proximate bladder carcinogens in nonindustrial-related bladder cancer. They also suggest that different etiological factors are involved in the pathogenesis of aggressive and nonaggressive bladder cancer.

INTRODUCTION

Uroepithelial tumors are one of the best characterized examples in which carcinogens have been linked to tumorigenesis. Studies in experimental animal models and humans have established that exposure to a number of compounds, particularly aryamines, will subsequently induce uroepithelial (bladder or ureter) carcinoma in a proportion of individuals exposed (1-3). However, not all individuals are equally susceptible to a similar dose, and such variability in response has been attributed to the complexity in the mechanism of carcinogenesis (3). For example, an aryamine such as benzidine (an antioxidant used in the dye industry) is a procarcinogen which must be converted to a carcinogen via a complex series of events, including N-hydroxylation and glucuronidation within the liver, renal tubular secretion to urine, and finally, hydrolysis in an acid medium to release a reactive nitrenium ion as the proximate carcinogen (4). Alternatively, aryamines can be metabolized by N-acetyltransferase to the arylacetylamine which is not a bladder carcinogen (5). It has been suggested that variations in the relative efficiency of alternative routes of drug metabolism result in variations in the amount of proximate carcinogen produced from a given environmental exposure, with some routes of metabolism conferring enhanced risk and others providing protection from carcinogenesis (6).

Even though certain specific environmental agents have been linked with bladder tumor formation in humans, the frequency of tumors for which a known carcinogen is involved is small and even in heavily industrialized areas is not more than 30% (7, 8). In the majority of patients who develop bladder cancer, no such etiology can be established. However, similar mechanisms may be involved with as yet unidentified chemicals in the environment acting as procarcinogens. The major difficulty in testing this hypothesis is that the nature of the environmental toxin is not known, much less its individual routes of metabolism.

A number of studies have compared the frequency distribution of acetylator phenotypes in bladder cancer populations with that in normal subjects (6, 9-11). The rationale for this approach has been that (a) acetylation might confer a protective route of metabolism, and (b) the phenotypic trait for fast or slow acetylation for one substrate is shared by other substrates that are metabolized by this route within an individual (12). Thus, phenotyping individuals with one substrate will determine that subject's ability to acetylate other drugs. Results from this approach have ranged from no difference between study groups to small significant differences associated with a low relative risk. Collectively, the results have suggested that slow acetylation confers a small difference in the development of bladder cancer (11), presumably by allowing a greater proportion of the procarcinogen to be converted to the proximate carcinogen.

The efficiency of other routes of drug metabolism could also alter risk of carcinogenesis, either conferring a protective effect by more rapid clearing of carcinogen, or increasing risk through more efficient conversion of procarcinogen to carcinogen. Routes of oxidative drug metabolism are generally undertaken by mixed function monoxygenase enzymes. These constitute a family of isozymes, each with its own substrate specificity (13). In instances in which a single isozyme is responsible for an individual metabolic route of a drug, the efficiency of conversion of parent drug to that metabolite can be used to provide a measure of the activity of the isozyme present. A well-characterized example is the isozyme responsible for the hydroxylation of debrisoquine (14, 15). A single form of cytochrome P-450 has been identified in human liver, which is responsible for more than 90% of the conversion of debrisoquine to 4-hydroxydebrisoquine (16). The activity of this enzyme is genetically determined by a single pair of genes with a homozygous recessive trait resulting in defective metabolism (17). This isozyme will metabolize only certain substrates, presumably due to structural configuration limitations, but such selectivity is fairly broad since a large number of drugs have now been identified which are metabolized by the debrisoquine
isozyme (18). It, therefore, provides a probe for one cytochrome P-450 isozyme which could be responsible for conversion of environmental procarcinogens to carcinogens.

Mephenytoin provides a marker substrate for another, genetically independent, isozyme of cytochrome P-450 (19). The isozyme responsible for the hydroxylation of S-mephenytoin has considerable substrate specificity as judged by 2 orders of magnitude difference in activity in metabolizing S-mephenytoin in comparison to R-mephenytoin (20) and the limited number of substrates that are metabolized by this enzyme (18). Thus, hydroxylation of S-mephenytoin provides a marker for a second isozyme of cytochrome P-450.

In many of the studies of genetic polymorphism of oxidative drug metabolism, the approach used has been to categorize a population as either poor or extensive metabolizers for the route of metabolism under investigation, recognizing that this overlooks the extensive interindividual variation in metabolism within each group. As such variability may contribute to the variation in risk, the approach used in the present study was to use indices of drug-metabolizing activity that linearly relate to drug-metabolizing activity and relate these as continuous variables to the risk of developing bladder cancer using logistic regression analysis.

Our objective was to use a case-control design, in which we controlled for potential bladder cancer risk factors such as age, sex, smoking habit, alcohol intake, and acetylator phenotype to study the association of the efficiency of two routes of oxidative metabolism with the risk of developing bladder cancer. The specific hypothesis tested was that the efficiency in oxidative metabolism of debrisoquine and mephenytoin is different in patients with bladder cancer compared to control subjects.

PATIENTS AND METHODS

Patients with uroepithelial cancer and a control population were drawn from the greater Bristol area in the southwest region of England with a source population of approximately 1,200,000. Each patient with carcinoma had his or her tumor visualized at cystoscopy and had histological confirmation of transitional cell carcinoma at the time of the drug metabolism study. Histological grading of tumors permitted classification as aggressive (i.e., Stage III, as defined by pleomorphic cell structure and evidence of invasion into submucosa and muscle) or nonaggressive (Stages I and II, as defined by a better differentiated cell structure and lesser extent of invasion) (21). Histological grading was performed on a blinded basis on which the pathologist had no knowledge of the results of the drug metabolism studies.

Choice of an appropriate control population is a crucial aspect of the methodology of noninterventional studies. The older age of presentation of most patients with bladder cancer precluded the conventional use of hospital workers and staff near the hospital as a control group. However, any older age group might be biased by selection for a specific disease entity and might include subjects with a unidentified bladder cancer. We chose to use patients referred to the same urological clinic for evaluation for whom there was definitive evidence that bladder cancer was not present. Approximately one-third of these subjects had no urological abnormality found, one-third had hyperplasia of the prostate, and one-third had miscellaneous urological problems. None had malignancy elsewhere. This control group was drawn from the same population of risk as the bladder cancer population and had similar socioeconomic and work-related background. The control group was of comparable age and sex and had a urological evaluation in which intravenous urography, urine cytology, and cystoscopy had failed to demonstrate evidence of bladder tumor. No subject in either the bladder cancer or control groups had a history of industrial exposure to known bladder carcinogens. Subjects with renal, hepatic, or cardiac disease were excluded, as were patients who were receiving medications which are known to influence drug metabolism, including barbiturates and cimetidine. The only drugs being taken at the time of the study were diuretics, digoxin, and aspirin. All other therapy was discontinued 48 h before the study.

Each subject had a detailed history with respect to possible environmental exposure to bladder carcinogens, smoking habit, and alcohol intake and a detailed urological investigation. Participating subjects gave their informed consent to the protocol which had been approved by the ethical committees in both institutions. Subsequently, fasting subjects received debrisoquine (10 mg) and racemic mephenytoin (100 mg) as a combined oral formulation (courtesy of Dr. A. Küpfer, Bern, Switzerland), and urine was collected over the subsequent 8 h. Food was permitted after 2 h. Urine volume was measured, and two 10-ml aliquots were stored at —20°C. One wk later, each subject received 100 mg dapsone. Eight h after p.o. administration, a 10-ml blood sample was collected, and the plasma was separated and stored at —20°C. All samples were transported frozen from Bristol to Vanderbilt University for subsequent analysis.

Debrisoquine (DB) and 4-hydroxydebrisoquine (4-OH-DB) were measured by electron-capture gas chromatography after derivatization with hexafluoroacetone (19). In studies defining the genetic polymorphism of debrisoquin hydroxylation, it has been customary to present the 5-mephenytoin recovery ratio (MR) as this mathematically permits ready identification of poor metabolizers.

\[ MR = \frac{DB}{4-OH-DB} \]

However, the metabolic ratio has the disadvantage of not being linearly related to the efficiency of drug-metabolizing activity. The data have, therefore, been analyzed using the transformed variable, the 8-h urinary recovery ratio (RR), as a phenotypic trait that is proportional to drug-metabolizing activity and more closely relates to fractional metabolic clearance.

\[ RR = \frac{4-OH-DB}{4-OH-DB + DB} \]

The phenotypic trait used in previous studies of genetic polymorphism of S-mephenytoin hydroxylation has been the 8-h urine S/R ratio of mephenytoin in urine (19). This has also been a way of identifying EMs* and PMs; however, this index does not linearly relate to drug-metabolizing activity. In contrast, the inverse (R/S) ratio does relate proportionally to drug-metabolizing activity (22). We have, therefore, measured the 8-h urinary enantiomeric (R/S) ratio of mephenytoin using stereoselective capillary gas chromatography (19).

Monoacetyldapsone and dapsone were measured using high-performance liquid chromatography with N-propylidesine as an internal standard (23). A slow acetylator phenotype was defined as a ratio of monoacetyldapsone to dapsone of 0.4 or less, and a rapid acetylator was defined as having a ratio greater than 0.4 (24).

Statistical Methods. Differences in the distribution of phenotypic indices of metabolism in the cancer and control groups are presented graphically as normit plots. These are normal probability plot descriptions of a cumulative distribution in which the Y-axis has been rescaled by application of the inverse normal transformation. If the index follows a Gaussian distribution, the points should fall on a straight line (25, 26). The normit plots also can provide a visual indication of multiple populations which are characterized by a gap in the curve produced by the data points.

Logistic regression (27–30) was used to test the association between indices of metabolic efficiency and risk of bladder cancer while controlling for other patient characteristics. With this method, the probability of experiencing the outcome, expressed as the natural logarithm of the odds ratio, is assumed to be a linear function of the factors of interest as well as the confounding factors. That is,

\[ \ln \left( \frac{E(Y)}{1 - E(Y)} \right) = B_0 + B_1Z_1 + \ldots + B_kZ_k \]

where, for a given subject,

\[ Y = \begin{cases} 0 & \text{did not experience outcome} \\ 1 & \text{did experience outcome} \end{cases} \]

* The abbreviations used are: EM, extensive metabolizer; PM, poor metabolizer.
and $E(Y)$ denotes the expected or average value of $Y$ for a defined population; $B_0$ is the base-line risk; $Z_1, \ldots, Z_k$ are the values of the covariants for the patient; $B_1, \ldots, B_k$ are the expression coefficients. These describe the amount by which risk, expressed as the natural logarithm of the odds ratio, changes when the covariate changes.

In performing a logistic regression, the coefficients are estimated by a maximum likelihood procedure (27). Each coefficient is compared with an estimate of its standard error to assess statistical significance. If the covariate has only two values (i.e., 1 and 0), the estimated coefficient has a further interpretation as the natural logarithm of the relative risk of experiencing the outcome. It is assumed that, in a case-control study, the exposure odds ratio is a good estimate of the relative risk (29).

To construct models which included only those covariates associated with changed bladder cancer risk, we utilized stepwise logistic regression with backwards elimination (30). Interaction terms were included in potential models. The BMDP statistical program, LR (30), was utilized for all computations.

**RESULTS**

Ninety-five patients with bladder cancer participated in this study at the time of their diagnosis and histological evaluation. Of these patients, 62 had nonaggressive tumors and the remaining 33 had aggressive tumors. Patients with bladder cancer were compared to a control group of 110 patients. Descriptions of the sex distribution, age, smoking habits, and alcohol consumption and the proportion of slow acetylators for these three subject groups are provided in Table 1. In this population, the patients with bladder cancer were primarily male (70%) and had a mean age close to 70 years; the controls were comparable. Neither smoking nor alcohol intake was a significant risk factor when occurring alone. However, smoking and alcohol interacted synergistically to substantially increase the risk of bladder cancer. In the cancer patients, 40% both smoked and drank as opposed to 20% of controls. Using logistic regression analysis to control for age and sex, this difference was statistically significant for both nonaggressive ($P < 0.001$) and aggressive ($P < 0.05$) tumors and conferred relative risk ratios of 5.0 and 3.6, respectively. After controlling for age, sex, smoking, and alcohol intake, the slow acetylator phenotype was not associated with increased risk of bladder cancer in this population (Table 1; $P > 0.2$).

The cumulative distribution of the 8-h urinary debrisoquine recovery ratio is presented as a normit plot in Fig. 1. In normal subjects, the PMs formed a discrete population with values below 0.12, while EMs had values greater than 0.12. In patients with aggressive bladder cancer, the frequency distribution was markedly shifted to the right, indicating a predominance of extensive debrisoquine hydroxylation activity. Only four patients with aggressive bladder cancer had a debrisoquine recovery ratio that was less than the mean of the recovery ratio in the control group, and only one was just within the PM range. The strong association of extensive debrisoquine metabolism with aggressive bladder cancer was confirmed by logistic regression analysis, which controlled for age, sex, smoking habit, alcohol intake, and acetylation and mephenytoin phenotypes (Table 2; $P = 0.006$). There was no evidence for an interaction between extensive debrisoquine hydroxylation and slow acetylation.

![Fig. 1. Normit plot for debrisoquine recovery ratios in normal subjects (O) and patients with nonaggressive bladder cancer (O) (top) and aggressive bladder cancer (O) (bottom).](image)
DRUG-METABOLIZING ACTIVITY IN BLADDER CANCER

A similar significant association was also found when the more commonly used debrisoquine phenotype, the debrisoquine metabolic ratio, instead of the recovery ratio, was used in a frequency distribution histogram rather than a normit plot (Fig. 2). In this instance, rapid metabolizers have smaller ratios (left), and slower metabolizers have higher ratios (right). There are clearly fewer slow metabolizers in the aggressive bladder cancer group.

To determine whether the difference in debrisoquine polymorphism between aggressive bladder cancer and controls was the result of a smaller than expected proportion of debrisoquine PMs among the patients with aggressive tumors, we performed a separate analysis which excluded PMs from both groups. Within this subgroup, the normit plot still showed an apparent association of aggressive bladder cancer with increased debrisoquine metabolism (Fig. 3), a finding which was confirmed by the logistic regression analysis (Table 2; P = 0.017).

In contrast to patients with aggressive bladder cancer, patients with nonaggressive bladder cancer had a similar cumulative frequency distribution of the debrisoquine recovery ratio to the control group (Fig. 1). This apparent lack of association was confirmed by logistic regression analysis (Table 2).

The cumulative distribution of the 8-h urinary R/S ratio of mephenytoin is presented as a normit plot in Fig. 4. In normal subjects, there were only two individuals who were PMs of mephenytoin as defined by an R/S ratio approaching unity. The normit plots of the distribution of the R/S mephenytoin ratio showed that patients with nonaggressive tumors were slightly more likely to be faster metabolizers than were the controls (Fig. 4). Logistic regression analysis confirmed a weak but significant association (Table 2; P = 0.04). In contrast, after controlling for sex, age, smoking habit, alcohol intake, acetylator phenotype, and debrisoquine recovery ratio, there was no difference in the cumulative frequency distribution of the mephenytoin phenotype between the patients with aggressive tumors and normal subjects (Fig. 4), and this index was not associated with increased relative risk (Table 2).

To illustrate the relative contributions of the smoking-alcohol interaction and the two routes of oxidative metabolism to the risk of developing a bladder tumor, we performed a logistic regression analysis in which each subject was classified as being above or below the median obtained from the control group for the debrisoquine recovery ratio and the mephenytoin R/S ratio. From this analysis, we estimated the relative risk of bladder cancer associated with: (a) the combination of a smoking habit and alcohol intake; (b) a debrisoquine recovery ratio above the median of the control group; and (c) a mephenytoin R/S ratio above the median of the control group. Consistent with previous analyses, the relative risk associated with the smoking-alcohol interaction was high (about 4) and similar for both aggressive and nonaggressive tumors (Fig. 5). For nonaggressive tumors, the risk associated with mephenytoin R/S ratios above the median of the control group was relatively weak (about 1.5). However, for aggressive tumors, the relative risk for patients with a debrisoquine recovery ratio above the median was in the same order of the risk associated with smoking-alcohol (about 4). Each of these relative risks was independent of each other.

DISCUSSION

We have found that the polymorphic distribution of the efficiency of two individual isozymes of cytochrome P-450 is
Fig. 5. Relative risk of developing cancer associated with combined smoking habit and alcohol consumption (3) rapid debrisoquine metabolism (33) and rapid mephenytoin metabolism (31) in nonaggressive (left) and aggressive (right) bladder cancer.

different in patients with bladder cancer in comparison to a control population. This observation is consistent with the hypothesis that conversion of as yet unidentified environmental procarcinogens can be undertaken by these mixed-function oxidase enzymes to form proximate carcinogens responsible for nonindustrial-related, sporadically occurring bladder cancer. There are, however, alternative explanations which require consideration. These include the possibility of selection bias in the control group of subjects, tumor-initiated increases in debrisoquine metabolic activity, and genetic linkage between oncogenes related to the bladder cancer and genes determining debrisoquine activity.

The difference in the distribution of the debrisoquine trait between the control group and patients with aggressive bladder cancer is a new observation. A previous study in which all patients with bladder cancer were presented together failed to show a similar difference (31). However, patients with aggressive and nonaggressive cancers were not differentiated. As aggressive tumors only account for one-third of the total, it is possible that differences in frequency distribution of the debrisoquine phenotype in the aggressive subjects were not observed due to the presence of a larger population of nonaggressive bladder cancer in whom no such difference occurs. It is also of interest that the extensive debrisoquine metabolism in the aggressive cancer group is similar to that noted in a group of lung cancer patients (32). The frequency distribution profile of the debrisoquine metabolic ratio (Fig. 2) shows a marked similarity in the comparison between control and aggressive bladder cancer subjects to the comparison of controls and lung cancer subjects. None of the patients in the present series had coexisting lung cancer, and no instance of coexisting lung and bladder cancer was reported in the lung cancer study. This suggests that efficient debrisoquine metabolism may be associated with at least two sites of carcinogenesis.

The magnitude of the risk associated with extensive debrisoquine metabolism, assessed as the debrisoquine recovery ratio, in developing aggressive bladder cancer was considerable, conferring a 4-fold increase. This can be contrasted to the risk of being a slow or fast acetylator, the only previously suggested risk factor related to drug metabolism. In studies examining slow acetylation polymorphism as a risk factor in bladder cancer, relative risk ratios have varied from only 1.01 to 1.67 (6, 9-11), with the highest risk ratios occurring in subjects exposed to known bladder carcinogens (9, 33). The population of this study provided one of the higher values when considered by univariate analysis (Table 1). However, this was no longer a significant risk factor when a multivariate analysis included other significant risk factors. Our inability to find statistical significance may be due to the small size of the study sample, but even if significant, the presence of the isozyme of cytochrome P-450 responsible for debrisoquine metabolism appears to be more important than slow or rapid N-acetylation in the development of aggressive bladder cancer.

In contrast to aggressive bladder cancer, the frequency distribution of the debrisoquine trait was similar in patients with nonaggressive bladder cancer to controls. However, in this instance there was evidence that rapid metabolism of mephenytoin provided a weak but independent risk factor. Even though this phenotype contributed a lesser relative risk, it does emphasize that etiological factors differ between aggressive and nonaggressive bladder cancer and supports the suggestion based on differential clinical progression that these are separate pathological entities (34, 35). These differences provide a rational but not entirely reliable basis for using histopathology obtained at the time of diagnosis to predict the patient's prognosis (21). However, surprisingly little attention has been given to differentiating different risk factors in these two entities. It is relevant to note, however, that industry-related bladder cancer is usually an aggressive tumor (1).

Previous large scale epidemiological studies have suggested that, in addition to industrial environmental exposure, smoking constitutes a risk factor in the development of bladder cancer (36, 37). An association between smoking and slow acetylation in bladder cancer (38) has suggested an interaction between inhaled carcinogens and individual routes of drug metabolism. We have also previously demonstrated a synergy in risk with combined smoking and alcohol consumption in the present population (39). This interaction was statistically independent of the two oxidative polymorphic relationships to bladder cancer and occurred equally in both nonaggressive and aggressive bladder cancer. This would suggest that neither the debrisoquine nor mephenytoin isozymes of cytochrome P-450 are involved in the production of proximate carcinogens from cigarette smoke.

One explanation for the differences observed between our populations is bias introduced by our selection of a control population. From a clinical perspective, the control population appeared to be similar to the cancer population in all respects apart from their urological diagnosis. There was no difference in associated diseases or drug intake, and no patient was debilitated from his or her cancer at the time of the study. The control population is unlikely to be biased by another disease entity as a variety of urological conditions were present, and one-third had no abnormality found. Furthermore, results between controls and nonaggressive bladder cancer were similar and were comparable to values obtained in previous populations obtained in younger, disease-free, normal populations (19). These findings suggest that the control population is representative of a normal population and does not provide a source of bias.

Another explanation is that it is potentially feasible for aggressive bladder cancer to stimulate debrisoquine metabolic activity. There is currently no precedent for a tumor-associated increase in drug-metabolizing activity, and this increase would have to be selective as mephenytoin metabolism is not altered. The more usual change associated with hepatic metabolism is a decrease in activity associated with hepatic metastases and loss of liver mass. However, no patient had clinically demonstrable hepatic metastases at the time of the study. It is not possible to evaluate bladder cancer patients before the time of diagnosis. However, we are initiating a study to examine the
41. Evans, D. A. P., Eze, L. C., and Whibley, E. J. The association of the slow
Genetic Predisposition to Bladder Cancer: Ability to Hydroxylate Debrisoquine and Mephenytoin as Risk Factors

Amir Kaisary, Patrick Smith, Evelyne Jacq, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/47/20/5488

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.