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DNA HYPERPLOIDY AS A MARKER FOR BIOLOGICAL RESPONSE TO BLADDER CARCINOGEN EXPOSURE

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A marker for biological response to bladder carcinogen exposure was evaluated in a cross-sectional study of 504 workers at high risk due to a range of exposures to various carcinogenic aromatic amines, primarily 2-naphthylamine. A quantitative fluorescence cytology method using the DNA-binding dye, acridine orange, was employed to measure DNA in exfoliated urothelial cells. DNA hyperploidy (>5 C) was observed in 16 (21.6%) of 74 workers who had been exposed compared with 15 (3.5%) of 430 workers who had not ($p < 0.001$). The prevalence of DNA hyperploidy increased in a dose-response manner from 3.5% to 60% with increasing duration of exposure. The association between DNA hyperploidy and exposure persisted when adjustment was made for age and cigarette smoking ($p = 0.0001$). The prevalence of the marker was greatest for exposed workers who smoked (23%), and lowest for those who had no exposure and who had not smoked (2%). This study indicates that DNA hyperploidy can serve as a marker for identifying workers who are at increased risk in occupational groups exposed to bladder carcinogens.

The epidemiologic identification of numerous cohorts of workers potentially exposed to bladder carcinogens emphasizes the need for early markers to identify bladder cancer risk in individual cohort members (Hueper, 1969; Johnson and Parnes, 1979; Schulte *et al.*, 1985*b*). Currently, occupational bladder cancer surveillance programs are costly, suffer from a lack of sensitive and specific tests, and often involve invasive diagnostic procedures (Cartwright, 1984). More specific markers for early recognition of bladder cancer risk could possibly alleviate the need for costly and invasive diagnostic procedures in many subjects, and more closely target those individuals who should undergo further evaluation.

We have used two cytometric methods to measure the absolute nuclear fluorescence intensity (ANFI), and thereby the DNA content, in individual cells: simple filter microfluorometry (SFM) (Hemstreet *et al.*, 1983) and, more recently, quantitative fluorescence image analysis (QFIA) (Bass *et al.*, 1987). With both methods, the biochemical composition of each cell can be measured relative to its optical image. With respect to bladder cancer, the particular strength of these ANFI methods is their demonstrated ability to detect low-grade tumors with greater sensitivity than routine (Papanicolaou) cytology (Hemstreet *et al.*, 1983, 1984; Bass *et al.*, 1987) and to subclassify atypical cells associated with malignancy.

In 1981, a study to notify and screen a cohort of 1,385 chemical production workers exposed to aromatic amines, primarily 2-naphthylamine, was initiated to assess the predictive value of DNA hyperploidy determined by quantitation of the ANFI of exfoliated urothelial cells as a marker for biological response to exposure to bladder cancer risk factors. The objectives, organization and results of other aspects of this program have been reported (Schulte *et al.*, 1985*a*,*c*, 1986). Fourteen cases of bladder cancer—4 times the number of cases expected—have now been observed in the cohort. Specifically, the study now reported aimed to determine (a) the extent to which DNA hyperploidy is a sensitive and specific marker for biological response to etiological factors for bladder cancer, and (b) whether the ANFI methodology is a valid, relatively non-invasive means by which to identify DNA hyperploidy.

METHODS

Members of the cohort were notified by mail in 1981 of their potentially high risk for bladder cancer and invited to participate voluntarily in a medical screening program (Schulte *et al.*, 1985*a*,*c*, 1986). Initial screening included: medical, occupational and risk-factor history; physical examination; urinalysis for hematuria and pyuria; routine urine cytology; and ANFI urine cytology by SFM. Individuals with a history (based upon job category) of high exposure to aromatic amines or whose physical examination and/or test results were abnormal or suggestive of bladder cancer also underwent a diagnostic urologic evaluation. The extent of each evaluation was determined by the clinical judgment of the attending urologist. This study was not designed as a controlled trial, but all samples for laboratory analysis were blind-coded.

The results reported are based upon the medical examination of 504 cohort members who had at least initial bladder cancer screening that included ANFI urine cytology and whose exposure status, based on job category, was known. The cohort originally consisted of 1,385 workers, 798 of whom were alive and residing within 50 miles of the screening site. Of this eligible group, 39 were seeing private physicians and chose not to participate in the study. A total of 545 people responded positively and did, in fact, participate in the screening program, but specimens from 22 people in the initial screen could not be evaluated for ANFI due to poor preservation, loss, or destruction in shipping, while exposure information based on job categories was not available for 19 of the 523 individuals who had ANFI evaluation.

In order to assess the value of DNA hyperploidy as a marker for biological response to exposure, the relationships between ANFI results and various risk factors were determined. The risk factors evaluated were occupational exposure to aromatic amines, age, ethnic group, sex, and cigarette smoking. Occupational exposure was defined in terms of job category (either "exposed" or "unexposed") and duration of employment. Duration of employment was used as a surrogate for exposure since many workers who had undergone exposure to aromatic amines were not in the job categories designated as "exposed". Cigarette smoking was measured in pack-years. Methods for defining and measuring exposures have been described (Schulte *et al.*, 1985*a*,*c*, 1986).

Absolute nuclear fluorescence intensity (ANFI) DNA measurements

Samples containing exfoliated urothelial cells were preserved immediately by adding an equal volume of buffered

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50% non-fluorescing ethanol and were then refrigerated or placed on ice pending transport to the laboratory. After thorough mixing, each sample was divided into 2 equal parts for ANFI analysis and routine cytology. Samples were analyzed within 3 weeks of collection. Aliquots for routine urine cytology were prepared by cyto-preparatory techniques using Millipore filters.

Staining with acridine orange (AO) dye and scanning using a Leitz MPV microscope were performed according to Hemstreet *et al.* (1983) and West *et al.* (1987). At least 10 cells per sample were selected for simple filter microfluorophotometer (SFM) measurements, although a great many more were actually scanned by the cytologist. The brightest and most atypical-looking cells were selected for quantitation of DNA content. A sample was scored ANFI-positive for hyperploidy if a single cell was found with DNA content of >5 C; otherwise the sample was scored ANFI-negative. The threshold of 5 C (0.5 phosphor particle units) was established on the basis of our earlier work using the SFM method (Hemstreet *et al.*, 1983) and confirmed by the QFIA method (Bass *et al.*, 1987).

Statistical analysis

DNA measurements of AO-stained urothelial cells were evaluated for their relationships to measures of exposure to 2-naphthylamine and cigarette smoking, and for age (at examination) and other risk factors, using multiple logistic regression analysis (Cornfield, 1951). Odds ratios were calculated for the relationship between DNA measurements and various risk factors (Snedecor and Cochran, 1967; Kleinbaum *et al.*, 1982). The 95% confidence intervals for each odds ratio were calculated using the Taylor series approximation (Kleinbaum *et al.*, 1982). Chi-square tests were used to assess trends in the data (Lilienfeld *et al.*, 1956). Differences in mean pack-years of cigarette smoking, age and duration of employment were evaluated using Student's *t*-test (Brownlee, 1965). Specificity and negative predictive value of the test in relation to exposure to 2-naphthylamine were calculated (Morrison, 1979). In order to identify exposure, personnel records were reviewed and employees were classified by job category as either "exposed" or "unexposed" according to the job's potential for exposure to 2-naphthylamine.

RESULTS

Figure 1 shows the distribution of test results among the 504 subjects who had ANFI urine cytology (and job category-exposure information available), using the test with the highest C value when more than one sample per subject was analyzed. Samples from 31 (6% of 504) individuals contained one or more DNA-hyperploid (>5 C) cells. When the population was classified by occupational exposure to aromatic amines, based upon job category, the rates of positive ANFI results were 21.6% of the 74 exposed individuals, and 3.5% of the 430 unexposed workers ($p < 0.001$). The odds ratio of this association is 7.6 (95% CI, 3.4, 16.2) (Table I).

The effect of age was evaluated by comparing response curves in the exposed and unexposed groups at different ages (Fig. 2). For all ages, response was greater in the exposed group than in the unexposed group; however, all confidence intervals overlapped. The association between a positive response and age was significant for the unexposed group ($\chi^2 = 12.04$, 4 degrees of freedom, $p = 0.017$), but not for the exposed group ($\chi^2 = 3.54$, 4 degrees of freedom, $p = 0.47$). The mean age in the exposed group was 49.6 years and in the unexposed group 46.7 years; however, for subjects with ANFI-positive results, the mean age was 51.1 years in the exposed group and 52.2 years in the unexposed group. The unexposed group showed very little variation with age. Within the exposed group there was no statistically significant difference in the age distribution between individuals with ANFI-positive and ANFI-negative results. In the unexposed group, the difference was statistically significant ($p = 0.04$), workers having positive results being slightly older than those with negative results.

The rates of ANFI-positive results were 7.7% in Blacks and 3.7% in Whites ($p = 0.08$). None of the 26 women, all of whom were unexposed, was ANFI-positive, compared with 31 of 478 men ($p = 0.17$).

TABLE I - ANFI RESULTS BY JOB CATEGORY. N = 504

Job category	ANFI[1] positive	ANFI[1] negative	OR[2] (CI)[3]
Exposed	16	58	7.6 (3.6, 16.2)
Unexposed	15	415	
Total	31	473	

[1]Absolute nuclear fluorescence intensity.-[2]Odds ratio compared with lowest exposure duration.-[3]CI = 95% Confidence Interval.

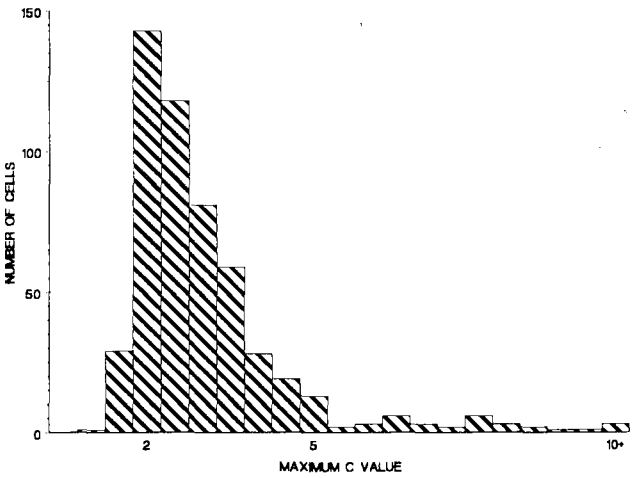

FIGURE 1 - Distribution of ANFI results among subjects screened.

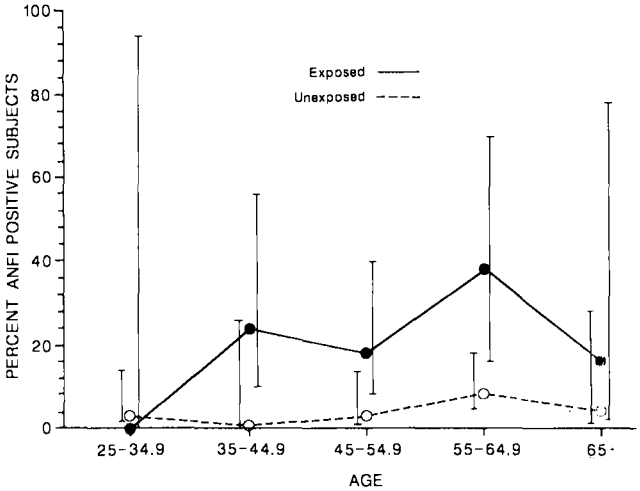

FIGURE 2 - The relationship between age and DNA hyperploidy among the exposed and unexposed subjects. Percent of subjects with >5 C DNA results are indicated by points on solid (exposed) and broken (unexposed) horizontal lines. Confidence intervals for each age group are indicated by vertical lines.

TABLE II - ANFI RESULTS BY DURATION OF EXPOSURE (YEARS OF EMPLOYMENT). N[4] = 332

Years of employment	ANFI[1] positive	ANFI[1] negative	OR[2] (CI)[3]
<1	8	222	—
1-10.9	12	76	4.4 (1.7, 11.1)
11-19.9	2	7	7.9 (1.4, 44.2)
>20	3	2	41.6 (6.1, 284.7)
Total	25	307	5.5 (2.2, 13.2)

[1]Absolute nuclear fluorescence intensity.-[2]Odds ratio compared with lowest exposure duration.-[3]CI = 95% Confidence Interval.-[4]No "duration of exposure" information available on 172 subjects who had ANFI.

A dose-response relationship was observed between duration of occupational exposure and positive ANFI results. The following rates of positive results were observed as exposure duration increased: 0–0.9 years, 3.5%; 1–10.9 years, 13.6%; 11–19.9 years, 22.2%; and >20 years, 60.0%. The odds ratios for ANFI for each duration of exposure category are shown in Table II. Each is statistically significant. Subjects with positive ANFI had had a longer mean duration of employment, 5.2 ± 7.8 years, than those with negative ANFI, 1.5 ± 3.4 years ($p = 0.02$). The age when first hired was higher in the positive group, 30.2 ± 10.4 years, than in the negative group, 26.4 ± 7.9 years, but this difference was not statistically significant ($p = 0.09$). The 2 groups had comparable lengths of time since first hire until the study date, with means of 21.9 and 23.5 years, respectively.

Cigarette smoking, another risk factor for bladder cancer, was also evaluated; 6.8% (24/350) of smokers were positive compared with 4.8% (6/124) of non-smokers; odds ratio = 1.44 (95% CI, 0.93, 3.6). The group with positive ANFI had a mean of 21.7 pack-years, compared with 17.2 pack-years for the group with negative ANFI measurements, but the difference was not statistically significant. The prevalence of positive ANFI was highest, 23%, for persons with 2 risk factors, exposure and smoking, and decreased to 2% for persons with neither risk factor (Table III). When the data were stratified according to occupational exposure the odds ratios of the association between ANFI and smoking were 1.8 (95% CI, 0.12, 26.7) in the exposed group and 1.8 (95% CI, 0.14, 21.9) in the unexposed group when smoking was defined as "current" or "never". When the definition of smoking was "current" or "former" and "never", the odds ratios were similar, 3.1 (95% CI, 0.14, 68.4) for the exposed and 11.7 (95% CI, 0.35, 385.9) for the unexposed. When the data were stratified by any smoking history the odds ratios for the association between exposure and positive ANFI were 6.6 (95% CI, 3.0, 14.3) for the smoking group and 11.1 (95% CI, 2.5, 48.2) for the non-smoking group. The combined effects of age, occupational exposure, and cigarette smoking were assessed in a logistic regression model. Only exposure was significant [OR = 7.6 (95% CI, 3.4, 16.7); $p = 0.0001$]. When duration of employment was included in the model, only this was significant; the adjusted odds ratio, when age and cigarette smoking were controlled for, was 1.1 (95% CI, 1.04, 1.2).

TABLE III - PREVALENCE OF ANFI[1]-POSITIVE RESULTS AMONG SCREENED WORKERS ACCORDING TO RISK FACTORS. N[2] = 474

Risk factors	Percent positive	Number positive/total
Exposure + smoking	23%	11/48
Exposure + no smoking	18%	4/22
No exposure + smoking	4%	13/302
No exposure + no smoking	2%	2/102

[1]Absolute nuclear fluorescence intensity.-[2]Smoking history not available for 30 subjects.

DISCUSSION

This study indicates that DNA hyperploidy, identified by a positive ANFI, is a marker for biological response to bladder carcinogen exposure. The finding of hyperploid cells (>5 C DNA) is associated with occupational exposure to aromatic amines, and there is a consistent dose-response relationship. As the duration of exposure increases, so does the prevalence of DNA hyperploidy. The association exists between DNA hyperploidy and occupational exposure when the effects of age and cigarette smoking are controlled for simultaneously ($p = 0.0001$).

The frequency of positive ANFI in the groups with both risk factors, occupational exposure and smoking, is 11 times the frequency for those with neither risk factor, but when the odds ratios are calculated for the association of smoking with ANFI, when stratifying for exposure, the confidence intervals encompass 1.0 even though the direction of the findings is consistent with an interactive effect. The appearance of the greatest prevalence of DNA hyperploidy in persons with both occupational and smoking exposures, and of lower prevalences in those with one or no risk factor(s) are consistent with what would be predicted on the basis of the relative potency of the risk factors (Lilienfeld *et al.*, 1956; Morrison, 1979; Matanoski, 1983). On the other hand, when stratifying for cigarette smoking, the association between exposure and positive ANFI is slightly greater in the unexposed group although the confidence intervals overlap.

Another critical test of whether ANFI is a useful marker for biological response to exposure is the extent to which non-exposed individuals test negatively. From the data in Table I, we calculated approximations of the specificity and negative predictive values for this test, which represented 96.7% and 87.7% respectively. These test parameters are only estimates of the true values. They are based on the assumption that all non-exposed individuals are negative for DNA hyperploidy. In fact, some small percent of the non-exposed individuals may be positive for DNA hyperploidy as a result of other non-occupational exposures. The negative test status is also time-specific since DNA hyperploidy does not develop with every exposure or at the same time after exposure. Hence, non-exposed individuals may test negative because they are evaluated at the "wrong" biological time, when the natural history of this condition is in a phase that is not detectable with current technology. For use in screening occupational populations where the prevalence of a condition is low, the specificity (and high negative predictive value) become quite important. The ANFI was specific and useful in this regard.

In this study, ANFI is not a surrogate for occupational exposure to aromatic amines; rather it is a marker for biological response to exposure. Differences in exposure and host characteristics influence the biological response that occurs in each individual. One host characteristic that may be of significant importance is the genetically-controlled ability to acetylate aromatic amines through the N-acetyltransferase pathway in the liver (Cartwright *et al.*, 1982; Lower, 1979).

We have demonstrated that the quantitation of absolute nuclear fluorescence intensity (ANFI), when applied under highly defined conditions with appropriate instrument calibration, appears to represent a promising and significantly improved marker for: (a) biological response to exposure to bladder carcinogens, and (b) clinical bladder cancer (response). In contrast to the situation with symptomatic patients, the final step to prove a clinical cancer outcome in this cohort will require years—and expanded screening programs. However, the implications of using DNA hyperploidy determined by quantitation of absolute nuclear fluorescence intensity as a marker for biological response in high-risk groups are that it

may serve: (1) to identify an earlier point for intervention such as chemo-prevention or pre-clinical chemotherapy; (2) as a basis for distinguishing those who may need more aggressive, invasive and costly diagnostic procedures such as cystoscopy, IVP, or biopsy; and (3) as a non-invasive means for routine monitoring of previously diagnosed patients at high risk for recurrence, patients with hematuria, and men in the over-50 age-group in whom a 3.9% incidence of genito-urinary cancer has been identified (Messing *et al.*, 1987).

High-risk occupational cohorts, patients with hematuria, and men over 50 are valuable resource groups for screening with QFIA to elucidate the immunologic and biochemical changes that occur in the oncogenic process. The point at which DNA hyperploidy occurs in the carcinogenic process is not clear, although a positive ANFI was observed in one case in this study 2 years prior to the histologic confirmation of malignancy. Results of animal and human studies by others (Levi *et al.*, 1969; Tribukait, 1984; Auer and Zetterberg, 1984; Naslund *et al.*, 1987) investigating the role of DNA hyperploidy in carcinogenesis would suggest the likelihood that many of the patients with positive ANFI results are experiencing biochemical changes months or years in advance of clinically manifest disease. DNA changes have been observed in symptomatic patients prior to biopsy-confirmed clinical disease (deVere White *et al.*, 1988). Further study of the sequence of events from initiation to the manifestation of symptomatic disease may provide information about other markers for identifying individuals at high risk for cancer. The exact relationship of these changes to DNA ploidy, histopathology and oncogene activation remains to be investigated. Future studies in other cohorts will aid in the development of clinically useful tests that employ non-invasive sampling for very early cancer detection, identification of intermediate endpoints for possible chemo-preventive or chemotherapeutic intervention, and improved patient management.

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