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Micronuclei in epithelial cells from sputum of uranium workers

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LOOMIS DP, SHY SM, ALLEN JW, SACCOMANNO G. Micronuclei in epithelial cells from sputum of uranium workers. *Scand J Work Environ Health* 1990;16:355-62. The cytogenetic effects of exposure to radon progeny and cigarette smoke were assessed with the exfoliated-cell micronucleus assay among 99 uranium workers. Cells with micronuclei were determined in one sputum specimen from each worker. Exposure to radon progeny and smoking habits were classified from interview data collected at the same time as the sputum specimens. Underground miners were considered exposed to radon progeny, and the other workers were considered unexposed. Neither radon progeny exposure nor cigarette smoking had any appreciable effect on the prevalence of cells with micronuclei; the crude prevalence ratios for the two groups were 1.0 (95 % confidence interval 0.7-1.4) and 0.9 (95 % confidence interval 0.6-1.3), respectively. The effects of radon and smoking were not confounded by each other or by age, nor were they synergistic. These findings cast doubt on the use of sputum-based micronucleus assay in epidemiologic studies of other populations exposed to occupational or environmental lung carcinogens.

Key terms: biological markers, chromosomes, cytogenetics, lung cancer, mining, occupational health, radon.

The exfoliated-cell micronucleus assay is a relatively new cytogenetic technique for monitoring the effects of exposure to carcinogens and mutagens. Micronuclei are small bodies containing deoxyribonucleic acid (DNA) in cell cytoplasm. They are formed from chromosomes or chromosome fragments which lag behind at anaphase and are not incorporated into a daughter nucleus during cell division (1). An increased frequency of micronuclei indicates an increased frequency of structural and/or numerical chromosome aberrations (2). The micronucleus assay can be performed on exfoliated epithelial cell populations as a measure of the frequency of chromosome aberrations in dividing stem cells of epithelial tissues (3). Exfoliated epithelial cells can generally be sampled without invasive techniques. Thus the need to use surrogate tissues like blood to measure genetic effects can be eliminated when the exposure of interest is primarily associated with tumors in epithelial tissues, such as the lung, oral cavity, esophagus, or urinary bladder (4). This capability is particularly useful because many occupational and environmental agents induce tumors at epithelial sites. Since cell culturing is not required, the assay can be performed on fixed tissue specimens, and it is there-

fore possible to conduct retrospective studies using archival materials (3).

Previous research has shown that the prevalence of exfoliated oral epithelial cells with micronuclei is increased by exposure to cigarette smoke, smokeless tobacco, and ionizing radiation (3, 5) and decreased by dietary supplementation with retinoids (4). Micronucleated cells have also been shown to be increased in the sputum of smokers (6). However, despite the importance of occupationally and environmentally induced lung cancer, there has been relatively little work either in the laboratory or in the field to develop the exfoliated-cell micronucleus assay as a tool for investigating such exposure-disease relationships.

In this study, we used the exfoliated-cell micronucleus technique to examine historical sputum specimens of uranium industry workers, including underground uranium miners, from the Colorado plateau area of the United States. This application provided a field test for use in epidemiologic studies of the micronucleus analysis of exfoliated epithelial cells in sputum to detect genetic damage potentially related to lung cancer. Colorado plateau uranium workers are a promising population for such a test because (i) underground uranium miners suffer from a marked, well-documented, dose-dependent increase in mortality from lung cancer as a result of their exposure to radon progeny (7-10), (ii) important confounders and effect modifiers in the radon-lung cancer relationship have been identified (7, 11, 12), (iii) quantitative, cell-level dosimetric models have been elaborated (13), and (iv) cells exfoliated from the bronchial epithelium can be easily obtained in sputum, which has been collected from these workers, examined, and archived since the 1950s. This population was of interest beyond the op-

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portunity it offered for testing the micronucleus technique because studies of uranium workers have contributed significantly to the understanding of the pathophysiology of lung cancer (14) and, in addition, because of the potentially large public health consequences of environmental exposure to radon progeny (15).

Epidemiologic studies of underground uranium miners have shown that the incidence of lung cancer increases with the level of cumulative exposure to radon progeny and that lung cancer incidence among smoking uranium miners is greater than the sum of that among individuals exposed to smoking alone or radon progeny alone (11). Our goal in this investigation was to determine (i) if the prevalence of micronucleated cells is higher for underground uranium miners than for workers not exposed to radon progeny and if the prevalence increases with the level of exposure among miners and (ii) if exposure to both radon progeny and cigarette smoke synergistically increases the prevalence of micronucleated cells.

Materials and methods

Subjects and biological materials

The subjects for this study were selected from a group of employees from underground and open-pit uranium mines and uranium mills in the Colorado plateau region of the United States (US). These workers had participated in a workplace sputum cytology screening program begun by the US Public Health Service in 1957 and conducted under the supervision of G Saccomanno since that time (16). The data collected in this program are not fully computerized, so exact numbers and descriptive characteristics of the screened population were not available, but an estimated 17 700 workers have submitted at least one sputum specimen since the program's inception.

Beginning in 1963, screening was conducted annually at uranium facilities throughout the region, and all workers, including blue-collar and white-collar occupational groups, were asked to participate. Refusal was rare. The participants were administered a questionnaire which solicited information on present and past occupations and smoking patterns, and they submitted a sputum sample for later examination. A saline aerosol inhalant was used to induce sputum production in most of the subjects. Sputum was collected in a 50 % alcohol fixative, blended and centrifuged to yield a uniform concentrate of cellular material (17), and then smeared on microscope slides, allowed to air dry, and stained with a standard "Papanicolaou" stain. The slides were evaluated for evidence of cancer or squamous metaplasia, and the results were recorded.

The following method was used to select the subjects for the study. First, a pool of workers employed during the period of interest was identified by ran-

dom sampling from a master list of approximately 34 000 sputum specimens collected from 1964 to 1988. (Previous years were excluded because slides produced by methods introduced in mid-1963 were more quantitatively representative of the entire specimen and easier to read than earlier ones.) Workers with complete data on occupation, length of employment, and smoking at the time the sputum specimen was collected were considered eligible for inclusion if, in addition, (i) their sputum specimen contained alveolar macrophages indicating the presence of pulmonary cells, (ii) their cytodiagnosis did not indicate cancer, (iii) those whose occupation was underground uranium mining had mined for at least six months before the sputum examination, and (iv) smokers had smoked for at least six months prior to the sputum examination. Finally, we screened the sputum slides from these eligible subjects with a light microscope equipped with objectives with magnifications of 40 X and 100 X to assess their suitability for micronucleus analysis. Only individuals were included whose sputum smear contained sufficient numbers of cells of adequate morphologic clarity to allow micronuclei to be reliably identified. A target of 100 subjects was set as the size of the population on the basis of feasibility considerations and preliminary power estimates, and the sampling procedure was repeated until a sufficient number of slides had been selected for analysis.

Micronucleus assay

The selected slides were treated in xylene to remove the coverslips; then successive baths in absolute alcohol and 1 N hydrochloric acid were used to remove the original Papanicolaou stain. The slides were then restained with the Feulgen reaction (18) and scored for the frequency of micronucleated cells. An experienced cytologist, blinded with respect to the occupational and smoking history of the subjects, scored one slide in its entirety for each subject, with the goal of counting at least 1000 cells/subject. Epithelial cells of squamous, columnar, metaplastic, and dysplastic morphological types were included in the counts and their relative frequencies were determined. Obscured and degenerated cells were excluded, as were cells with pycnosis, condensed chromatin, karyoclasia, and karyolysis; however, the number of cells with the last four kinds of anomalies was noted. To be classified as a micronucleus, an extranuclear body had to meet the following criteria, used previously in our laboratory: (i) membrane bound, (ii) no more than one-third the size of the nucleus, (iii) same focal plane as the nucleus, and (iv) color, staining intensity, and texture similar to the nucleus (19). Further details of the assay methods are available from the first author upon request. We assessed measurement reliability by having the same observer blindly reread a 10 % random sample of the study slides.

Exposure data

Data on radon progeny exposure, smoking, and other variables were taken from the screening questionnaires associated with each sputum specimen. Workers whose occupation at the time of the screening was underground uranium mining were classified as exposed to radon progeny, and those with other occupations were treated as unexposed. Similarly, only individuals who said they smoked at the time of the screening were classified as smokers, and all others were classified as non-smokers. The intensity of smoking exposure was additionally described for cigarette smokers by the number of cigarettes smoked per day.

No quantitative data were available on the level of exposure to radon progeny at the time each worker was screened, so the number of hours spent underground each day was used as a surrogate measure of exposure level. The workers were also classified as to whether they were screened before or after 1971, since underground radon concentrations were reduced by federal government regulations introduced in that year.

Statistical analysis

The analysis of the data focused on the effect of radon progeny exposure and smoking on the prevalence of micronucleated cells. We calculated crude prevalence ratios contrasting the average prevalence of the exposed and unexposed groups by taking the ratio of the mean prevalence per 1000 cells, $\hat{y}$, in each group [ie, $\hat{y}_i = \Sigma [1000 (y_{ij}/n_{ij})]/N_j$, where y_{ij} and n_{ij} indicate the number of cells with micronuclei and the total number of cells counted, respectively, for each individual, and N_j is the number of individuals in exposure group j]; 95 % confidence intervals were calculated for the prevalence ratios as standard large-sample confidence intervals for a ratio of means:

$$\exp \{ \ln PR \pm 1.96 [\text{var} (\ln PR)]^{1/2} \}$$

estimating $\text{var} (\ln PR)$ as $[v_1/\hat{y}_1^2 + v_0/\hat{y}_0^2]$, where $j = 1$ if exposed and 0 if unexposed, $v_j = [\text{se} (\hat{y}_j)]^2$, and PR = prevalence ratio. Weighted least-squares regression and logistic regression were used to obtain the estimates for the prevalence ratio adjusted for covariates.

The effect of exposure to radon progeny and tobacco smoke on the distribution of the individual prevalence of micronucleated cells was examined in a second series of analyses. Each subject was classified with respect to whether he fell above the 50th and 75th percentiles of the distribution (2 and 3 micronucleated cells/1000 cells, respectively), forming groups of "cases" with elevated prevalence and "noncases" without it. The association between exposure and individual prevalences greater than the specified percentiles was estimated by the odds ratio, and 95 % confidence intervals were computed by the test-based method (20). Adjusted odds ratio estimates were obtained by logistic regression. The SAS (statistical analysis system) system was used for all the analyses (21).

Results

Descriptive results

A total of 341 workers was drawn from the master list of 1964–1988 sputum specimens. Fifty-three were considered ineligible for the study because of missing or incomplete data ($N = 35$), no alveolar macrophages ($N = 13$), or less than six months of exposure ($N = 5$). Sputum slides from 166 of the remaining 288 individuals were judged amenable to micronucleus analysis when examined by microscopy. One hundred and twenty-two slides were considered unusable, primarily because thick or very uneven smears ($N = 35$), excessive mucus content ($N = 35$), or large numbers of inflammatory cells ($N = 17$) obscured epithelial cells or because the number of epithelial cells was insufficient ($N = 26$). The first 105 eligible subjects with acceptable sputum slides were designated as the study group to meet the planned sample size of 100 subjects, allowing for the possibility of subsequent losses. Six individuals were later found to have missing smoking or occupational data and were dropped from the study, leaving a final study group of 99 subjects.

The study group was essentially the same as the original sample of 288 eligible workers with regard to exposure to underground uranium mining, smoking, age, and year of screening. Table 1 gives the descriptive characteristics of the study group by mining and smoking exposure.

Table 1. Characteristics of the study group by current underground uranium mining and smoking status.

	Total number	Males		Mean age (years)	Mean years in industry	Mean calendar year of specimen	Mean hours underground/day	Mean years mined	Former miners		Cigarette smokers				Pipe or cigar smokers		Former smokers	
		N	%						N	%	Mean cigarettes/day	Mean years smoked	N	%	N	%	N	%
Miners																		
Smokers	25	25	100	36	10	1973	/	7	.	.	22	88	16	13	3	12	.	.
Nonsmokers	22	22	100	36	9	1971	7	8	.	.	.	.	.	4	.	.	6	28
Nonminers																		
Smokers	30	29	97	31	4	1976	.	1	4	13	28	93	21	11	2	7	.	.
Nonsmokers	22	22	100	37	6	1976	.	1	2	9	.	.	.	5	.	.	7	32

A total of 92 450 epithelial cells was counted and 190 micronucleated cells were found, yielding an average of 994 cells counted per subject (range 222–1628), and an overall mean prevalence of 1.92 micronucleated

Figure 1. Distribution of micronucleated cells (MNC) among the workers by mining and smoking status.

Table 2. Crude relationships of mean number of micronucleated cells/1000 cells to mining, smoking, and age. (SE = standard error, PR = prevalence ratio, 95 % CI = 95 % confidence interval)

Exposure	N	Mean	SE	PR	95 % CI
Mining					
Nonminers ^a	52	2.01	0.27	1.0	-
All miners	47	2.09	0.27	1.0	0.7–1.4
< 8 h/d ^b	13	1.60	0.32	0.8	0.5–1.3
8 h/d	34	2.27	0.35	1.1	0.5–2.3
After regulation ^c	26	2.21	0.36	1.1	0.7–2.2
Before regulation	21	1.94	0.42	1.0	0.6–1.6
Smoking					
Nonsmokers ^a	44	2.12	0.32	1.0	-
All smokers	55	1.99	0.23	0.9	0.6–1.3
1–19/d ^d	12	2.85	0.57	1.3	0.8–2.1
20–29/d	29	1.96	0.33	0.9	0.6–1.4
≥ 30/d	9	1.10	0.22	0.5	0.3–0.8
Age (years)					
20–39 ^a	71	2.11	0.23	1.0	-
40–59	26	1.82	0.36	0.9	0.6–1.4
≥ 60	2	2.94	1.64	1.4	0.5–4.3

^a Reference category.

^b Hours underground per day; reference group = nonminers.

^c Year of sputum specimen in relation to introduction of radon concentration standard in 1971; reference group = nonminers.

^d Number of cigarettes per day (includes cigarette smokers only); reference group = nonsmokers.

cells/1000 cells. The distribution of micronucleated cells, shown by mining and smoking status of the worker in figure 1, was skewed, with a median of approximately two micronucleated cells and a 75th percentile of approximately three micronucleated cells. Twenty-two subjects had no micronucleated cells, and one individual (a nonsmoking nonminer) had 11 micronucleated cells.

Ten slides were blindly reread so that the reliability could be assessed. Five had exactly the same number of micronuclei on both readings, and four others differed by one micronucleus. However, the estimated prevalence of micronucleated cells was different on the first and second readings for every slide because of variation in the total number of cells counted. The mean difference of -0.09 micronucleated cells/1000 cells was not significant ($P = 0.81$) in a paired *t*-test.

Mining and smoking effects

The crude prevalence ratios and their 95 % confidence intervals for the effects of underground uranium mining, smoking, and age on the mean prevalence of micronucleated cells are shown in table 2. Exposure to radon progeny had little effect, regardless of daily time underground or the introduction of controls on radon progeny exposures. Smoking also had little effect overall or among the majority of cigarette smokers, who consumed between 20 and 29 cigarettes/day, but the prevalence was slightly increased among light smokers [prevalence ratio (PR) 1.3, 95 % confidence interval (95 % CI) 0.8–2.1] and decreased among the heaviest smokers (PR 0.5, 95 % CI 0.3–0.8). The prevalence was also slightly elevated among the oldest workers (≥ 60 years old), but that group contained only two individuals.

The effects of separate and combined exposure to radon progeny and tobacco smoke on the mean prevalence of micronucleated cells are shown in table 3. When compared with the prevalence of micronucleated cells in the group with no exposure to radon progeny or tobacco smoke, the prevalence of micronucleated cells was reduced among individuals with exposure to only one agent. Exposure to radon and smoking together had no effect.

Table 3. Crude effects of separate and combined exposure to radon progeny and smoking on mean number of micronucleated cells/1000 cells. (SE = standard error, PR = prevalence ratio, 95 % CI = 95 % confidence interval)

Exposure ^a		N	Mean	SE	PR	95 % CI
Mining	Smoking					
-	- ^b	22	2.64	0.49	1.0	-
-	+	30	1.55	0.27	0.6	0.3–1.4
+	-	22	1.60	0.39	0.6	0.2–1.5
+	+	25	2.51	0.37	1.0	0.5–2.0

^a + and - indicate exposure present and absent, respectively.

^b Reference category.

The crude relationship of mining, smoking, and age to an elevated individual prevalence of micronucleated cells is shown in table 4. Few workers had greater than the median prevalence of micronucleated cells. Nevertheless, the results were generally similar to those for average prevalence (table 2) and therefore indicated little or no mining effect and a small inverse smoking effect, which became stronger with increasing cigarette consumption. Some effects appeared to become stronger among the workers with more than three micronucleated cells/1000 cells. But the number with such a high prevalence was small, and the odds ratios were correspondingly imprecise.

The crude associations of separate and combined exposure to mining and tobacco smoke with an elevated individual prevalence of micronucleated cells were also similar to those for the mean prevalence and suggested negative effects of exposure to only one agent and no effect from exposure to both.

None of the effect estimates from the preceding analyses were appreciably changed by adjustment for age and/or by adjustment of mining effects for smoking, or vice versa. Neither were they altered by the exclusion of former miners and former smokers from the analysis or by the deletion of the nonsmoking non-miner with 11 micronucleated cells. Repeat analyses restricted to the small, single micronuclei characteristic of chromosome breaks and to the subset of micronuclei which unambiguously met all of the scoring criteria also indicated no effect of either mining or smoking on the prevalence of micronucleated cells.

Discussion

In this study, we found no association between the prevalence of micronucleated epithelial cells in sputum and either exposure to radon progeny through employment in underground uranium mines or direct exposure to tobacco smoke. These results warrant careful examination since both radon progeny and tobacco smoke are human carcinogens and mutagens whose chromosome damaging effects are detectable with other cytogenetic methods.

There is abundant evidence that the micronucleus assay is sensitive to effects induced by both ionizing radiation and tobacco smoke (3, 5), and one study has shown an increased prevalence of micronucleated cells in sputum from smokers (6). Although the micronucleus analysis of sputum has not previously been tested as an indicator for radon progeny effects, the prevalence of structural chromosome aberrations in peripheral blood lymphocytes has been shown to be increased among underground uranium miners (22) and individuals with residential radon exposure (23, 24). These findings indicate that radon exposure induces chromosome breaks which should be detectable with the micronucleus end point. Furthermore, both dosimetry and the

Table 4. Crude relationships of the individual prevalence of micronucleated cells over a specified level to mining, smoking, and age. (OR = odds ratio, 95 % CI = confidence interval)

Exposure	Total number ex- posed	Individual prevalence of micro- nucleated cells					
		>2/1000 cells			>3/1000 cells		
		N ^a	OR	95 % CI	N ^a	OR	95 % CI
Mining							
Nonminers ^b	52	16	1.0	.	9	1.0	.
All miners	47	16	1.2	0.5—2.7	9	1.1	0.4—3.2
< 8 h/d ^c	13	4	1.0	.	0	0.0	.
8 h/d ^c	34	12	1.2	0.5—3.1	9	1.7	0.6—4.9
Preregulation ^d	26	10	1.4	0.5—3.8	5	1.1	0.3—3.9
Postregulation	21	6	0.9	0.3—2.8	4	1.1	0.3—4.2
Smoking							
Nonsmokers ^b	44	16	1.0	.	10	1.0	.
All smokers ^a	55	16	0.7	0.3—1.7	8	0.6	0.2—1.6
< 20	12	6	1.9	0.5—6.7	5	2.8	0.7—10.4
20—29	29	9	0.9	0.3—2.3	3	0.5	0.1—1.8
≥ 30	9	0	0.0	.	0	0.0	.
Age (years)							
20—39 ^b	71	23	1.0	.	12	1.0	.
40—59	26	8	0.9	0.4—2.5	5	1.2	0.4—3.7
≥ 60	2	1	2.1	0.1—33.6	1	5.9	0.4—66.5

^a N = number of exposed with more than the specified number of micronucleated cells/1000 cells.

^b Reference category.

^c Hours underground per day; reference group = nonminers.

^d Year of sputum specimen in relation to introduction of radon concentration standard in 1971; reference group = nonminers.

^e Number of cigarettes per day (includes cigarette smokers only); reference group = nonsmokers.

absence of hematopoietic tumors among uranium miners would seem to suggest that such cytogenetic damage might be the most prevalent in the respiratory epithelium. In light of these arguments, it is worthwhile to consider several aspects of the design and execution of this study and of the application of the micronucleus assay which may have led to spurious null results.

The inclusion of former underground miners and former smokers among the unexposed might have resulted in potentially biasing exposure misclassification if, contrary to expectation, either exposure had long-term effects on the prevalence of micronucleated cells. However, deleting these individuals from the analysis did not alter the results. A negative bias could also have arisen from exposure misclassification as a result of the categorization of open-pit uranium miners as unexposed, if such workers experienced above-background radon exposures and had an elevated prevalence of micronucleated cells as a result. The mean prevalence of micronucleated cells was actually lower for open-pit miners than for any other group, however. Some exposure misclassification is probably inherent in the use of screening interview data to determine mining and smoking status, however, and either nondifferential or differential exposure misclassification from this source could account for the overall null results. Misclassification may be greater for smoking than for current occupation, since the screening inter-

views took place at worksites, where the interviewer was allowed to determine the type of facility, whereas subject responses were the only source of smoking data.

Biased sampling is of particular concern in this study because of the inherently subjective nature of the procedure used to select slides amenable to micronucleus analysis from among those of all eligible subjects. The impact of the selection procedure on the estimated prevalence of micronucleated cells cannot be assessed directly, but there is no evidence that it produced appreciable imbalances in regard to measured variables likely to be causally related to micronuclei. Of course, other unmeasured selection factors might have led to distorted results, but since there are few known risk factors for an abnormal prevalence of micronucleated cells, no obvious candidates for such selection factors emerge.

The same difficulty arises in attempts to assess the potential for distortion by uncontrolled confounding. Occupational chemical exposures and environmental exposures to radon progeny or tobacco smoke are of concern as potential confounders. We had no data on these exposures, but the mean prevalence of micronucleated cells in all the exposure groups was similar to the "background" prevalences of 1.2 and 3 micronucleated cells/1000 cells previously reported in sputum of nonsmokers in other studies (6, 25). Few other variables appeared to warrant control as potential confounders.

Other concerns arise from conceptual and technical aspects of the exfoliated-cell micronucleus assay. Unbiased assessment of the frequency of chromosome-damaging events with this technique requires that the target tissue be sampled when one generation of cell division has occurred after the exposure of interest. Earlier or later sampling tends to dilute the exfoliated micronucleated cells and underestimate the frequency of damaging events in dividing cells. The optimal time window for sampling an epithelial tissue in relation to a point exposure is defined by the period required for exposed, dividing cells to complete one division and their daughter cells to migrate to the epithelial surface. Sampling time becomes less critical with a chronically-exposed population because continuous cell division supplies new micronuclei as long as the exposure persists. The cytokinetics of the human bronchial epithelium are poorly known, but rodent data suggest that several days are required for division and migration, and the entire epithelium turns over in a period ranging from two weeks to several months (26). The design of this study required individuals classified as exposed to have been mining or smoking at the time of sampling and for at least the preceding six months. Thus sampling time was unlikely to have had a major effect on the estimated frequency of micronucleated cells.

Aspects of cellular differentiation in the human bronchial epithelium may also have had an impact on

the study results. In contrast with the classical view that micronuclei observed in exfoliated bronchial cells are the product of a single division in the basal layer (4), there is evidence that several types of differentiated bronchial cells, in addition to the basal layer, are capable of division (27), and end-stage cells may arise from them through pathways involving one or two divisions (26).

Another interpretational difficulty with the assay system used in this study results from the constraints imposed by the nature of sputum as a medium for recovering bronchial cells. The investigator's limited ability to control the source of sputum and its cellular content is a particular concern in this regard. Although epithelial cells of all morphologic classes were included in the micronucleus analysis, 98 % of the cells counted were squamous cells, with the remaining 2 % composed of approximately equal numbers of ciliated columnar cells and metaplastic bronchial cells. Squamous cells are a common constituent of sputum (28) but are characteristic of the oral and pharyngeal epithelium and occur in the bronchi only as a result of metaplastic transformation after an injury (27). Thus it is possible that, as a result of the quantitative dominance of squamous cells, the micronucleus analysis may have yielded information largely about cytogenetic events in the upper respiratory tract and failed to capture the effects of inhaled radon progeny, which are manifested primarily in the epithelium of the bronchi (13).

In contrast to the effects known or dosimetrically predicted for radon progeny, tobacco smoking is demonstrably related to outcomes ranging from micronuclei (5, 6) to cancer (29) in the oral epithelium. Therefore the predominance of squamous cells alone is unlikely to explain the simultaneous lack of both radon and smoking effects. The smoking data may nevertheless suggest other pathways which could potentially bias toward null or negative effects. For example, the decline in the prevalence of micronucleated cells with increasing cigarette consumption (table 2) could have been generated by biased detection if our conservative scoring criteria resulted in micronucleated cells being differentially excluded among heavy smokers, perhaps because of their characteristically "dirtier" sputum.

The inherent heterogeneity of sputum may also have affected the results if it produced variability in the micronucleus counts which we were unable to measure, for example, between slides from the same specimen or between specimens from the same individual. Such variability might be large compared with the known component due to counting error. More complete analyses of variability are common in experimental studies (30), but generally not feasible in human observational studies like this one. If such error were present in the micronucleus data, it would, unlike errors in exposure classification, produce a spurious null prevalence ratio only if it were differential by exposure.

The assay results may also have been influenced by the age of the slides or by the restaining process used to prepare them for the micronucleus assay if either factor caused micronucleated cells to be lost or go unrecognized. We excluded any slides with evidence of physical deterioration (eg, severe fading, cracking) and encountered no technical difficulties in restaining. Although there was no direct evidence that age or processing affected the micronucleus counts, the possibility is difficult to assess and remains a potential source of error. As with other assay errors, however, differential cell losses would be required to eliminate a true effect of mining or smoking totally, although the statistical power to detect an effect could still be reduced by nonsystematic losses.

Concluding remarks

We used exfoliated-cell micronucleus analysis of routinely collected, historical sputum specimens to search for evidence of increased genetic damage in bronchial epithelial cells among workers exposed to radon progeny and tobacco smoke. We found no evidence of an association between an increase in the prevalence of cells with micronuclei and exposure to either factor, acting independently or in combination. These findings fail to support our initial hypotheses that the prevalence of micronucleated cells would be increased in a dose-dependent fashion by exposure to radon progeny and that radon and smoking exposures would act synergistically.

The null findings may represent the true state of nature, but given the contrast between the relatively well-developed theoretical and empirical basis for suspecting that both exposures induce cytogenetic and other effects detectable by sensitive methods, our results might most appropriately indicate that the micronucleus analysis of sputum is not yet ready for general application in epidemiologic research. Nevertheless, they point to the need for laboratory-based work to develop, validate, and standardize the exfoliated-cell micronucleus assay further for applications to a variety of human epithelial tissues. They reveal, in addition, the need for new methodological research on the epidemiologic use of biological markers which would explore such topics as the assessment of selection bias and the definition of confounding variables.

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