Genetic Risk Assessment in Cigarette Smokers

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INTRODUCTION

All over the world tobacco is consumed as cigarettes. Cigarette smoke continues to represent a challange to the biologists and chemists concerned with the effects on human health (De Marini, 1983) . Smoke produced by the burning end of the cigarette contains a mixture of more than 3800 identified compounds, some of which are biologically active (IARC, 1986).

There is a controversy with regard to the mutagenicity of tobacco smoke although experimental and epidemiological evidence indicates the carcinogenic effect of tobacco smoke (Doll and Peto, 1976; Wynder and Stellman, 1977; Benedict et al., 1984). Tobacco smoke is found to cause sister chromatid exchanges by some workers (Lambert et al., 1978; Obe and Herha, 1978; Bala Krishna Murthy, 1978; Hopkins and Evans, 1980; Carrano 1982, Parodi et al., 1982; Husum et al., 1982, Livingston and Fineman, 1983; Lundberg and Livingston, 1983; Wulf et al., 1983; Husgafvel-Pursianen, 1987; Kao-Shan et al, 1978; Ghosh and Ghosh, 1988; Reidy et al., 1988), whereas others (Hollander et al., 1978; Ardito et al., 1980; Crossen and Morgan, 1980; Hedner et al., 1983) did not find any effect of tobacco smoke on the frequency of SCE.

Some workers found tobacco smoke to cause chromosomal aberrations (Obe and Herha, 1978; Obe et al., 1982; Vijaylaxmi and Evans, 1982; Littlefield and Joiner, 1986). But no such results were found in other reports (Nordenson et al., 1978; Hurgafvel-Pursianen et al., 1980; Bendener et al., 1988, 1989). These authors, however, did observe an increase in the frequency of SCE.

Obe and Herha (1978), Obe et al. (1982,1984, 1994), Vijaylaxmi and Evans (1982) and Littlefield and Joiner (1986), however, found tobacco smoke to increase the frequency of CA as well as SCE.

To resolve the above controversy and as a sequel to the recommendations of International Commission For Protection Against Environmental Mutagens And Carcinogens (ICPEMC) (Bridges et al., 1979), the present study was undertaken. Since *in vitro* cytogenetic study is amongst one of the most important parameters to detect the risk to human health it was, therefore, considered worthwhile to conduct such a study on cigarette smokers.

Since chromosomal aberrations (CA) and sister chromatid exchanges (SCE) in metabolically competent cells are sensitive parameters for evaluating cigarette smoke genotoxicity (Doolittle et al., 1990) and are becoming increasingly used as *'biological response markers'* (Hagmar, 1990; Wogan, 1992; Perrera et al., 1992) for linking exposure to genotoxic agents with initiating events in the carcinogenesis process, these end points were taken as the main parameters, while mitotic index (MI) and satellite associations (SA) were taken as supporting ones.

MATERIALS AND METHODS

This study comprised 100 occupationally non-exposed male individuals in all, 50 cigarette smokers and 50 non-smokers matched with respect to age, sex and social status.

A survey was conducted using a proforma showing age, sex, number of cigarettes smoked per day and the duration of consumption of tobacco (in years).

Duplicate short term lymphocyte cultures were set up from heparinized blood according to the method of Moorhead et al. (1960) with minor modifications. Lymphocytes were cultured by adding 0.5 ml of blood in 5 ml of RPMI 1640 medium (Hi Media) supplemented with 20% foetal calf serum (Sigma), and 0.1 ml phytohaemagglutinin (Sigma), 10 μg/ml colchicine (Sigma) was added to the culture two hours prior to harvesting.

Lymphocytes were harvested after 48h for chromosomal aberrations (CA). Slides were prepared and stained with 4% Giemsa (E. Merck). For each person 100 well spread metaphases were analysed.

For mitotic index (MI), 5000 cells per individual were counted from Giemsa-stained slides. MI was calculated by using the formula

$$MI = \frac{\text{No. of dividing cells}}{\text{Total no of cells scored}} \times 100$$

For sister chromatid exchanges (SCE), 5-bromodexyuridine (Brdu) (Sigma) (10 ug/ml per culture) was added 24h after setting up of the cultures. Harvesting was done after 72h. Slides were prepared by air drying method and stained with solution of Hoechst 33258 (Sigma) (50 ug/ml in distilled water) and 4% Giemsa (E.Merck) following the method of Perry and Wolff (1974) with slight modifications. For calculating the frequency of SCE per cell, 25 metaphases per individual were analysed as per standard practice.

For evaluating the frequency of satellite associa-

tions (SA), 100 good metaphases were scanned. The criteria described by Hansson (1970) were followed for evaluating the SA. The criteria are (1) The satellite ends of the associating chromosomes had to be directed towards each other with their longitudinal axes meeting between their short arms, and (2) the distance between the centromeres of associated chromosomes should not exceed the total length of one 'G' chromosome, its satellite excluded. Screening of slides was done in double blind manner to obviate the risk of bias. The data obtained were statistically analysed applying the student's 't' test.

RESULTS

The survey revealed that most of the cigarette smokers complained of cough. Some of them also complained of respiratory-tract diseases. However, none of them suffered from lung cancer.

The data on various parameters obtained during the present investigation have been given in tables 1-6.

Much higher values of MI were observed in cigarette smokers (6.59) as compared to controls (3.78). The mitotic index showed elevation with the increased duration of consumption and the number of cigarettes consumed per day. A dose-effect relationship was apparent with regard to the duration as well as number of cigarettes smoked (Tables 1 and 2). The values are significant at $P < 0.01$.

The mean percentage of CA found in the smokers was 2.94 and that in the controls was 0.98 resulting in a highly significant difference at $P < 0.01$ (Table 3). Both chromosome type aberrations viz. dicentrics, rings, acentric fragments, chromosome gaps and chromosome breaks and chromatid type aberrations viz. chromatid gaps, chromatid breaks and isochromatid exchanges were encountered in the cigarette smokers. All the values for these aberrations were significant at $P < 0.0.1$. The cigarette smokers showed a regular increase in the values of CA with the duration of consumption (Table 4) and number of cigarettes smoked per day (Table 5). Maximum values of CA (5.83) and SCE (8.60) were reported in individuals who have been smoking for 16-20 yrs. Strangely, the controls showed only one dicentric, acentric fragment and chromosome gap (Table 3).

Table 4 shows the frequency of SCE in cigarette smokers and their controls. A significant difference was observed in the induction of SCE in smokers (7.67) as compared to controls (3.64). Tables 4 and 5 show significant elevation in the frequencies with the duration of smoking and the number of cigarettes smoked per day.

Table 6 shows the frequency of SA in smokers and their matched controls. Higher values of SA were observed in cigarette smokers (10.54) as compared to controls (5.54). Frequency of DG type associations was the maximum, while 3D type was the minimum. The values were highly significant ($P < 0.01$) in the smokers as compared to the controls.

DISCUSSION

Leuchtenberger and Leuchtenberger (1970) reported an alteration in mitotic cycle in mouse 3T3 cells that were exposed to the gas phase of cigarette smoke. These authors further found that smoke from cigarettes containing a mixture of tobacco and marijuana significantly increased the mitotic index (Leuchtenberger and Leuchtenberger, 1971). Korte et al. (1981) also recorded a high mitotic activity in smoke-treated Chinese Hamsters. However, a clear proof of significant increase in the mitotic index in the cultured human lymphocytes of nonsmokers and smokers was reported by Littlefield and Joiner (1986) who found that the mean MI varied from 2.8 in nonsmokers to 4.5 in the smokers, a significant increase. The reported increase in the mean MI from 3.78 to 6.59 in the present sample $P<0.01$) is in keeping with the above reports.

A significant effect of smoking was found both in the frequency of individual type of CA as well as total CA. It is in conformity with the earlier obser-

Table 1: Mitotic index(MI) in cigarette smokers and controls with duration of smoking

Duration (In years)	*Number of samples*	*Number of cells*	*Number of metaphases*	*MI Mean ±SD*
Control	50	243333	9396	3.78 ± 0.42
Smokers	50	238645	15729	6.59 ± 0.47*
0-5	8	38263	2337	6.10 ± 0.21
6-10	22	104734	6747	6.44 ± 0.42
11-15	14	67071	4635	6.91 ± 0.37
16-20	6	28577	2010	7.04 ± 0.18

* Significant at $P < 0.01$

Table 2: Mitotic index (MI) in cigarette smokers and controls with number of cigarettes consumed per day

Number of Cigarettes smoked per day	*Number of samples*	*Total Number of cells scored*	*Number of metaphases observed*	*MI Mean ±SD*
Control	50	243333	9396	3.78 ± 0.42
Smokers	50	238645	15729	6.59 ± 0.47*
6-10	20	95488	5902	6.17 ± 0.26
11-15	21	100225	6839	6.81 ± 0.38
16-20	9	42932	3009	6.97 ± 0.37

* Significant at P < 0.01

vations of Obe and Herha (1978), Vijaylaxmi and Evans (1982), Fredga et al. (1982), Obe et al. (1984), Littlefield and Joiner (1986) and Reuterwall et al. (1990). The dose-effect relationship reported by Huttner and Schoneich (1980) is also apparent in the present sample. Such a relationship was also recorded with regard to number of cigarettes smoked (Obe et al., 1982). Thus smoking definitely causes significant effect in the frequencies of both individual types of CA as well as total CA. Obe et al. (1994), however, could not find a significant effect of smoking in the frequency of individual types of CA, whereas they also reported significant increase in total CA. These authors also did not find any correlation with the mean number of cigarettes smoked

Table 3: Types of chromosomal aberrations in smokers and control individuals

Group	*Controls*	*Smokers*
No. of individuals	50	50
No. of metaphaes	5000	5000
Chromosome Type Aberration		
Dicentric	1(0.02)	10 (0.20)
Ring	-	4 (0.08)
Acentric Fragement	1(0.02)	12 (0.24)
Chromosome Gap	1(0.02)	4 (0.08)
Chromosome Break	-	4 (0.08)
Translocation	-	5 (0.10)
Total (without gaps)	2(0.04)	35 (0.70)
Chromatid Type Aberration		
Gaps	85(1.70)	139(2.78)
Breaks	47(0.94)	110(2.220)
Isochromatid Exchange	-	2(0.04)
Total (without gaps)	49(0.98)	112(2.24)
Total Chromsomal Aberrations	49(0.98)	147(2.94)

Values in parentheses indicate aberrations per 100 metaphases. All values are significant at p <0.01

Table 4: Frequency of chromosomal aberrations (CA) and sister chromatid exchanges (SCE) with duration of smoking

Duration (In years)	*Number of samples*	*CAs (Mean ± SD)*	*SCEs (Mean ± SD)*
Control	50	0.98 ± 0.71	3.64 ± 0.69
Smokers	50	2.94 ± 1.96*	7.67 ± 1.24*
0-5	8	1.12 ± 0.35	6.63 ± 0.72
6-10	22	2.04 ± 0.99	7.59 ± 1.11
11-15	14	4.14 ± 1.40	8.01 ± 1.24
16-20	6	5.83 ± 1.32	8.60 ± 1.40

* Significant at P < 0.01

Table 5: Frequency of chromosomal aberrations (CA) and sister chromatid exchanges (SCE) with no. of cigrates smoked per day.

Number of cigarettes per day	*Number of samples*	*CAs (Mean ± SD)*	*SCEs (Mean ± SD)*
Control	50	0.98 ± 0.71	3.64 ± 0.69
Smokers	50	2.94 ± 1.86*	7.67 ± 1.24*
6-10	20	1.75 ± 0.85	7.42 ± 1.14
11-15	21	3.19 ± 1.69	7.64 ± 1.10
16-20	9	4.77 ± 1.64	8.41 ± 1.45

* P < 0.01;

Table 6: Frequency of satellite associations observed in cigarette smokers and control individuals

	Number of Individuals	
	Control Individuals 50	*Cigarettee Smokers 50*
Total cells scanned	5000	5000
Types of Satellite Associations :		
DD	44 (0.88)	125 (2.50)
DG	154 (3.08)	229 (4.58)
GG	37 (0.74)	83 (1.66)
2 DG	17 (0.34)	49 (0.98)
2 GG	12 (0.24)	26 (0.52)
2 G 2D	13 (0.26)	21 (0.42)
3 D	6 (0.12)	8 (0.16)
Association Per cells	5.54 ± 2.50	10.54 ± 3.13

Values in parentheses indicate the SA per 100 metaphases
* Significant at P < 0.01

and the duration of smoking, whereas during present study a clear correlation was observed between these factors and the frequency of CA.

A number of investigations have been carried out with regard to the clastogenicity of cigarette smoke on the frequency of SCE in cultured human lymphocytes. As against a few reports not finding any effect of cigarette smoke on the SCE frequency (Hollander et al., 1978; Ardito et al., 1980; Crossen and Morgan, 1980; Hedner et al., 1983) a host of other investigators reported a significant increase in the frequency of SCE in smokers as compared to nonsmokers (Lambert et al., 1978; Bala Krishna Murthy, 1979; Hopkins and Evans, 1980; Carrano 1982; Parodi et al., 1982; Husum et al., 1982, Livingston and Fineman, 1983; Lundberg and Livingston, 1983; Kao-Shan et al, 1987; Ghosh and Ghosh, 1988; Wulf et al., 1983; Reidy et al., 1988; Hollander et al., 1978; Ardito et al., 1980; Crossen and Morgan, 1980; Hedner et al., 1983; Husgafvel-Pursianen et al., 1980 Reuter Wall, 1990). The present investigation has also shown a significant increase in the frequency of SCE in cigarette smokers.

The International Agency for Research on Cancer (IARC, 1986) evaluated as many as 27 studies and recorded 10.1 SCE/cell as the mean SCE frequency in smokers. Bender et al. (1988) in their study on the largest sample so far reported mean frequencies of 9.02 SCE/cell for smokers (N = 65) and 8.08 SCE/cell for nonsmokers (n = 220). In the present study the mean frequencies are 3.64 ± 0.69 in nonsmokers and 7.67 ± 1.24 in smokers, an increase of 4 SCE/cell, a quite big margin.

Two observations are common to most of the studies, viz. time-dose effect and the effect of sex on the frequency of SCE. Whereas time-dose-effect was quite apparent in the present sample also, the sample being male only the other parameter could not be studied. Opinions differ as to the effect of age. Whereas Bender et al (1988-1989) did not find any effect of age on the frequency of SCE, Sarto et al. (1985), Husum et al. (1986) and Lazutka et al. (1994) reported that the number of SCE increased with age. Although the present study was not carried out with this aspect in view, a cursory glance at the raw data (Table 1) does not show any increase in the number of SCE.

The cigarette smoke also showed synergistic effect in enhancing the frequency of CA and SCE in association with exposure to other genotoxicants (Nordenson et al., 1978; Husgafvel-Pursiainen et al., 1980; Bala Krishan Murthy and Prema 1979; Dewdney et al., 1986; Galloway et al., 1986; Wulf et al., 1986; Bendner et al., 1988; Bigatti et al., 1988; Anderson et al., 1988, 1991; Yadav and Kaushik, 1996, 1997; Yadav and Seth, 1998a,b).

A large variation in SA occurs in human population. The individual association patterns may be caused by genetic as well as by several other factors such as age, sex, polymorphism of nucleolar constriction, hormonal state etc. (Zankl and Zang, 1978; Nilsson et al., 1975; Hansson, 1979). In the present study two fold increase in the frequency of SA was observed in cigaratte smokers as compared to nonsmokers. The difference is significant and may lead to increased probability of chromosomal translocations. Regular increase in SA with the increased period of consumption of cigarettes and number of cigarettes smoked per day was observed. Similar results were reported earlier for grape garden workers occupationally exposed to different pesticides (Rita et al., 1987), for fertilizer plant workers exposed to SO_2 and NH_3 respectively (Yadav and Kaushik 1996, 1997), for NOx and PAH-exposed workers (Yadav and Seth, 1998a, 1998b) and for hookah and bidi smokers (Yadav and Thakur, 2000a, b). An observation common to all these studies is that the frequency of DG type association was highest, whereas 3 D type was lowest.

To conclude, the significantally increased values of the four parameters during present investigation prove the clastogenicity and the genotoxicity of cigarette smoke.

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KEY WORDS Peripheral Blood. Lymphocytes. Mitotic Index. Chromosomal Aberrations. Sister Chromatid Exchanges. Satellite Associations. Genotoxicity.

ABSTRACT The genotoxic effect of cigarette smoke was investigated on somatic chromosomes of fifty occupationally non-exposed male cigarette smokers. These were compared to an equal number of non-smokers matched with respect to age, sex and social status. The mitotic index (MI), chromosomal aberrations (CA), sister chromatid exchanges(SCE) and satellite associations (SA) were analysed. All the parameters showed a significant increase ($P<0.01$) in the smokers compared with controls: viz. MI (3.78-6.59), CA (0.98-2.94), SCE (3.64-7.67) and SA (5.54-10.54). A clear time-dose-effect relationship was observed. Cigarette smoke is, thus, both clastogenic and genotoxic for human beings.

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