Induction, persistence and modulation of cytogenetic alterations in cells of smoke-exposed mice

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In spite of the major role played by smoking tobacco in the epidemiology of chronic degenerative diseases, it is difficult to mimic the genotoxic and carcinogenic effects of this complex mixture in animal models. We undertook an experimental study evaluating the time-course induction, persistence and modulation of cytogenetic alterations induced in BDF1 mice exposed whole-body to mainstream cigarette smoke. The animals were divided into five groups, including: (i) 72 sham-exposed mice; (ii) 72 mice exposed to smoke for up to 3 weeks; (iii) 72 mice treated daily with the thiol N-acetylcysteine (NAC, 0.5 g/kg body weight) with drinking water; (iv) 72 mice exposed to smoke and treated daily with NAC, starting 5 days before exposure to smoke; and (v) 48 mice exposed to smoke and treated daily with NAC, starting 1 day after discontinuation of exposure to smoke. After 1, 2, 3, 4, 5, 6, 7, 10 and 14 weeks since the start of exposure to cigarette smoke, eight mice per group were killed, and cytogenetic parameters were evaluated. Exposure to smoke induced a high frequency of micronucleated and binucleated (BN) pulmonary alveolar macrophages, which persisted for at least 14 weeks. The frequency of micronuclei increased early in bone marrow polychromatic erythrocytes, but declined to background levels upon discontinuation of exposure to smoke. By comparison, their induction in circulating normochromatic erythrocytes (NCE) was slightly delayed, less intense but still significant, and persisting for an additional 3 weeks. Administration of NAC, throughout duration of the experiment, strongly inhibited the smoke-induced formation of micronuclei in alveolar macrophages and had some transiently significant effect on the induction of BN macrophages. NAC did not significantly decrease the smoke-induced formation of micronuclei in bone marrow cells, whereas it attenuated the formation of micronuclei in peripheral blood NCE. When given after discontinuation of exposure to cigarette smoke, NAC did not affect the cytogenetic alterations but normalized the altered bronchoalveolar lavage cellularity. The present data provide a detailed analysis of time-related cytogenetic alterations in smoke-exposed mice, both in the respiratory tract and at a systemic level, and show the effects of NAC on these parameters and on the pulmonary inflammatory response.

Introduction

There is sound evidence that cigarette smoke (CS) represents a major risk factor in the pathogenesis of cancers at various sites and of many other chronic degenerative diseases (1). Although a quite extensive literature covers the genotoxic and carcinogenic properties of individual CS components in experimental test systems, less information is available on genotoxicity and carcinogenicity of CS as a complex mixture, and it is difficult to reproduce these effects in animal models.

In particular, the assessment of clastogenic effects in CS-exposed rodents led to conflicting findings. In fact, negative results were reported in some laboratories, for instance in studies evaluating sister chromatid exchanges (SCE) and chromosomal aberrations (CA) in bone marrow cells of CS-exposed Chinese hamsters (2), SCE in peripheral blood lymphocytes from Wistar II rats exposed whole-body to mainstream CS (3), or SCE, CA and micronucleated (MN) bone marrow cells of Sprague–Dawley rats exposed nose-only to the mainstream smoke generated by either traditional cigarettes or cigarettes which heat but do not burn tobacco (4).

In contrast, several studies yielded convincingly positive results. Thus, an elevation of SCE, following nose-only exposure to CS, was observed in bone marrow cells of both BC3F1/Cum mice (5) and B6C3F1 mice (6). Both structural and numerical CA were enhanced in pulmonary alveolar macrophages (PAM) of Fisher 344/N rats exposed to CS, either nose-only or whole-body (7). A stimulation of MN polychromatic erythrocytes (PCE) was detected in the bone marrow of NMR1 mice receiving i.p. injections of a CS condensate (8), sidestream smoke being more potent than mainstream smoke in producing this effect (9). An increased frequency of MN and binucleated (BN) PAM was observed in CBA/H mice exposed whole-body to mainstream CS (10). In our laboratories, the whole-body exposure of BDF1 mice to mainstream CS resulted in an evident increase of MN bone marrow PCE (11) and MN normochromatic erythrocytes (NCE) in peripheral blood (12). In the same mouse strain, the transplacental passage of genotoxic components following exposure of pregnant animals to CS led to the formation of MN PCE in fetal liver as well as in the liver and peripheral blood of newborn mice (13). In addition, the whole-body exposure to mainstream CS resulted in an enhanced frequency of MN PAM and MN bone marrow PCE in Sprague–Dawley rats (14), BDx rats (15,16), and Balb/c and/or BDF1 mice (16).

We report herein the results of an extensive study evaluating the time-course induction and persistence, for up to 14 weeks, of cytogenetic alterations, including MN and BN PAM, MN bone marrow PCE and MN peripheral blood NCE, in BDF1 mice exposed whole-body to mainstream CS for 3 weeks. Groups of mice, either sham exposed or CS exposed, received the chemopreventive agent N-acetylcysteine (NAC) with drinking water either throughout duration of the experiment or after discontinuation of exposure to CS. Previous studies evaluated the ability of oral NAC to inhibit cytogenetic

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altered by a variety of genotoxic agents in rodent cells. In particular, NAC inhibited the formation of MN PAM in Sprague–Dawley rats receiving intra-tracheal instillations of benz[a]pyrene (17). Following whole-body exposure of the same rat strain to CS, NAC was very effective in inhibiting the formation of MN PAM, but had poor effects on BN PAM and failed to affect the formation of MN PCE in bone marrow (14). Similarly, NAC did not modulate the induction of MN PCE in the bone marrow of C57BL/6 mice treated with 7,12-dimethylbenz[a]anthracene by gavage (18). NAC was successful in attenuating the frequency of MN PCE in both bone marrow (19) and peripheral blood (20) of mice receiving i.v. injections of doxorubicin, and inhibited the time course formation of MN NCE in the peripheral blood of BALB/c mice receiving i.p. injections of urethane (21).

Materials and methods

Animals

A total of 336 female BDF1 (C57BL×DBA/2) mice (Animal Laboratory, National Centre of Oncology, Sofia, Bulgaria), aged 10 weeks and weighing 20–22 g, were used. The animals were housed in plastic cages (eight mice per cage) on sawdust bedding, and maintained on standard rodent chow and tap water ad libitum. The animal room temperature was 23 ± 2°C, with a relative humidity of 55%, and a 12 h day–night cycle. The housing and treatment of mice were in accordance with national and institutional guidelines.

Treatment groups

After 10 days of acclimatization, the mice were randomly divided into five groups. A total of 192 mice were exposed to cigarette smoke for up to 3 weeks, whereas 144 mice were kept for the same time interval in a similar environment, but in the absence of cigarette smoke. Within the latter group, 72 mice did not receive any treatment, and were identified as sham-exposed mice. Seventy-two untreated mice and 72 smoke-exposed mice were treated daily with NAC, starting 5 days before the first day of exposure to cigarette smoke, and continuing until killing of animals. An additional 48 smoke-exposed mice were treated daily with NAC, but starting 1 day after discontinuation of exposure to cigarette smoke. NAC was given in the form of a commercially available pharmacological preparation (Flumucil, Zambon, Vicenza, Italy), which was added to drinking water in order to achieve a calculated daily intake of 0.5 g/kg body weight. Since the NAC formulation used is stable in drinking water for at least 2 days at room temperature (unpublished data), the drug-containing water was changed every 2 days, and dilution of the drug in drinking water for at least 2 days at room temperature was tested by analysis of data (data not shown). Figure 1 shows at a glance the time-course of cytogenetic end-points in the various treatment groups.

Exposure to cigarette smoke

Whole-body exposure to mainstream cigarette smoke was obtained, as described previously (14), by using filter-tipped commercial cigarettes (Arda-Bulgartabac) that have a declared content of 31.5 mg tar and 1.6 mg nicotine. Briefly, each one of the groups of mice undergoing this treatment was placed in a 22.5 l sealed glass chamber that was subsequently filled by means of a 50 ml syringe with the mainstream smoke generated by one cigarette. The chamber was opened after 10 min and, after a 1–2 min interval needed to renew the air, filled again with fresh smoke for a total of six times, for up to 21 consecutive days. The concentration of total particulate matter in the exposure chamber was, on average, 533 mg/m³ air.

Evaluation of cytogenetic parameters

At weekly intervals, from week 1 to week 7 after starting exposure to cigarette smoke, and then on weeks 10 and 14, peripheral blood was collected from the lateral tail vein of the eight mice from each experimental group, which were then anesthesized with diethyl ether and killed by cervical dislocation. The frequency of MN cells at a magnification of 1000× was scored 1000 PAM, 500 PCE and 10 000 NCE in different slides. From each mouse, 20 000 NCE were scored for the frequency of MN PCE, which was virtually overlapping the PCE:NCE ratio, and 1000 PCE were scored for the presence of MN cells. All slides were examined as blind-coded samples by two readers, each one scoring 1000 PAM, 500 PCE and 10 000 NCE in different slides.

Statistical analyses

Comparison between groups were made by Student’s t-test for unpaired data and by means of ANOVA for repeated measurements.

Results

None of the performed treatments significantly affected either the body weight of mice or the PCE:NCE ratio in the bone marrow, which was consistently close to 1 in all experimental groups (data not shown).

Table 1 summarizes the results of cytogenetic analyses performed in BDF1 mice, as related to exposure to cigarette smoke and/or treatment with oral NAC. The statistical analysis of data is also provided. Figure 1 shows at a glance the time-course of cytogenetic end-points in the various treatment groups.

The frequency of MN cells in sham-exposed mice (Figure 1, open circles) was rather stable throughout the 14 weeks of the experiment, ranging between 2.3 and 3.0‰ in bone marrow PCE, between 1.4 and 1.9‰ in PAM, and between 0.6 and 0.8‰ in peripheral blood NCE. The background frequency of BN PAM ranged between 22.9 and 29.0‰.

The whole-body exposure of mice to mainstream cigarette smoke (Figure 1, full circles), for 3 weeks (shaded areas in Figure 1) resulted in significant alterations of all monitored parameters, but with different intensity and distinctive time trends. In particular, the enhancement of MN frequency in bone marrow PCE was the earliest event, and returned to background levels after discontinuation of treatment. The enhancement of MN frequency in peripheral blood NCE was also statistically significant after just 1 week of exposure, reached a plateau lasting 5 weeks, with a maximum 2.1-fold increase over controls, and then progressively declined to background levels. PAM alterations were even more pronounced and persistent. In fact, the enhancement of MN frequency became statistically significant after 2 weeks, and was followed by a plateau, whose levels were up to 3.2-fold higher than those observed in sham-exposed mice. This plateau lasted from week 2 to week 10 of the experiment, i.e. 7 weeks after withdrawal of exposure to smoke. Only at the last time check, at week 14, the frequency of MN PAM tended to decrease, but it still was significantly higher than in sham-exposed mice. The frequency of BN PAM was significantly enhanced since the first week, and thereafter progressively grew by reaching a plateau on the fourth week, which persisted until the end of the experiment. The maximum increase over controls was 3.3-fold.

The daily oral administration of NAC in drinking water throughout the period of the experiment (Figure 1, open triangles) did not affect the frequency of cytogenetic parameters per se, as compared with sham-exposed mice. In smoke-exposed mice, the continuous treatment with NAC, starting 5 days before the first day of exposure to smoke, mostly displayed protective effects on the monitored end-points (Figure 1, full triangles). In fact, NAC markedly decreased the frequency of MN peripheral blood NCE, which was virtually overlapping with the levels recorded in sham-exposed mice. NAC was also quite effective in inhibiting the formation of MN PAM.
Inhibition was statistically significant at all sampling points, and there was no significant difference between sham-exposed mice and smoke-exposed mice treated with NAC, although the two curves did not overlap (Figure 1). In addition, NAC delayed the formation of BN PAM, whose frequency, as compared with sham-exposed mice, became statistically significant after 3 weeks of exposure to smoke rather than after 1 week. NAC treatment, whether given before smoke exposure or after its cessation, returned the relative population of PMN thereby restoring the relative population of PAM. Other groups of mice were treated daily with oral NAC, but starting the day after the 3-week period of exposure to cigarette smoke (Figure 1, open squares), a time when the frequency of MN PCE in bone marrow had spontaneously returned to background levels. When given according to this post-exposure regimen, NAC failed to affect the smoke-induced cytogenetic alterations in PAM and peripheral blood NCE. However, when evaluated at week 14 of the experiment, it was observed that the cellularity of bronchoalveolar lavage was still altered by the previous exposure to cigarette smoke, 11 to 13 weeks earlier, and that the continuous treatment with NAC, even when given after smoke exposure cessation, was successful in normalizing the cellular formula. In particular, as shown in Table II, compared with sham-exposed mice, the previous exposure to smoke still determined a significant, almost 10-fold increase of polymorphonucleates (PMN), and an equivalent decrease of PAM. On the other hand, both NAC administration schedules significantly reduced the proportion of PMN thereby restoring the relative population of PAM. Unfortunately, after this evaluation was made, the slides with
Fig. 1. Time course of cytogenetic alterations in PAM, bone marrow PCE, and peripheral blood NCE of BDF1 mice exposed whole-body to cigarette smoke (shaded areas) and/or receiving oral NAC daily with drinking water according to two treatment schedules. See Table I for details and statistical analysis.

Table II. Cellularity of bronchoalveolar lavage in variously treated BDF1 mice (eight per group), 14 weeks after the start of the experiment

<table>
<thead>
<tr>
<th>Type of cell</th>
<th>Cellularity (%) in variously treated mice</th>
<th>Sham</th>
<th>NACa</th>
<th>Smokeb</th>
<th>Smoke + NACc</th>
<th>Smoke + NACd</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAM</td>
<td></td>
<td>95.8±0.8</td>
<td>96.9±2.8</td>
<td>54.7±21.6***</td>
<td>88.4±16.7**</td>
<td>90.7±12.8**</td>
</tr>
<tr>
<td>PMN</td>
<td></td>
<td>4.0±0.7</td>
<td>2.8±1.9</td>
<td>44.7±21.7***</td>
<td>11.3±16.8**</td>
<td>9.0±12.7**</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td></td>
<td>0.2±0.4</td>
<td>0.3±0.5</td>
<td>0.5±1.4</td>
<td>0.1±0.4</td>
<td>0.3±0.5</td>
</tr>
</tbody>
</table>

Statistical analysis (Student’s t-test for unpaired data): significantly increased as compared with the corresponding sham-exposed mice, at ***P < 0.001; significantly decreased as compared with the corresponding smoke-exposed mice, at **P < 0.01.

a0.5 g/kg body weight in drinking water.
bWhole-body exposure to mainstream cigarette smoke for 3 consecutive weeks (see Materials and methods for details).
cNAC administration started 5 days before the first day of exposure to cigarette smoke and continued until the end of the experiment.
dNAC administration started 1 day after discontinuing exposure to cigarette smoke and continued until the end of the experiment.
the bronchoalveolar lavage cells collected at the previous times were no longer available to explore the time course of this end point.

Discussion

The results of the present study provide first of all a detailed picture of the cytogenetic alterations produced by the whole-body exposure of BDF₁ mice to CS. These alterations were particularly intense in PAM, near the main portal of entry involved in this type of exposure, but were also evident and significant at the systemic level, i.e. in bone marrow PCE and peripheral blood NCE. Therefore, the present data support and extend the conclusions drawn in previous cytogenetic studies in CS-exposed rodents, performed both in our laboratory (11–15) and other laboratories (5–10), whereas they contrast with some negative data reported in the literature (2–4).

The differential intensity and distinctive time trends of the observed cytogenetic effects are likely to depend on several factors, including toxicokinetic features (first-pass effect), the turnover and lifespan of the investigated cells and the mechanisms involved in the investigated alterations.

PAM are expected to be subjected to a strong first-pass effect after whole-body exposure to CS. It is noteworthy that these cells not only have a very intense sweeping activity, but they are also equipped with the inducible machinery capable of metabolizing inhaled carcinogens (23,24). PAM are extremely long-lived cells (months to years), but their turnover is warranted by the intense removal from the alveolar spaces via the mucociliary escalator, accounting for a daily removal of 0.75×10⁶ cells in rats (25). In humans, with a total estimate of 23×10⁶ PAM filling the alveolar spaces (~50–100 PAM per alveolus) (26), 24–120×10⁶ PAM were evaluated to be removed daily (27). Accordingly, the daily turnover of PAM appears to be in the range of ~1–5%. Although this is just a tentative estimate in humans, it suggests a slow renewal of the PAM population and a very long half-life of these cells. This explains the relatively slow increase of cytogenetic alterations in PAM after exposure to CS, and their long persistence after cessation of exposure. These patterns were well evident in MN PAM, which are the expression of CS clastogenicity (28), and even more in BN PAM, which can be regarded as the result of a cytokinesis failure or block after completion of nuclear division, due to agents disrupting the cellular skeleton, thus creating a situation where the production of additional genetic changes becomes probable. Multinucleated PAM were found to be longer-lived than MN PAM also after exposure of mice to α-emitting nuclides (29), which is not surprising since MN PAM are the consequence of a chromosome damage or loss, whereas multinucleated PAM are likely to have at least one functional nucleus.

In contrast, the formation of MN PCE in bone marrow was less intense but more rapid. In previous studies, the top levels were reached 24 h after starting exposure to CS of both mice (11,12) and rats (14). In general, after exposure to a clastogen, the formation of MN PCE in bone marrow requires ~6 h. This lag period includes the time needed for toxicokinetics and metabolism, and for completion of the erythroblast cell cycle and extrusion of its nucleus (30). The plateau of MN PCE persisted throughout the 3 weeks of exposure to CS, after which the frequency of MN PCE quickly declined to background levels after discontinuation of exposure. The kinetics of these alterations reflect the very rapid turnover and extremely short lifespan of PCE, which last just 12–24 h in the bone marrow (30).

The CS-induced increase of MN NCE frequency in peripheral blood was even less intense, and its peak was slightly delayed as compared with MN PCE. However, this clastogenic effect persisted longer, being still significant for 3 weeks after discontinuation of exposure to CS. The time course of MN NCE elevation depends on the fact that, 12–24 h after migration of PCE from bone marrow to peripheral blood, they mature into NCE (30). Following acute treatments, MN NCE are excessively diluted in the pre-existing circulating NCE population, but, in case of long-lasting treatments, MN NCE accumulate to a significant extent (31), as it was the case for exposure to CS in the present study. As discussed below, alternative sources of MN NCE have been shown to occur. The observed persistence of CS-induced MN NCE is consistent with the fact that NCE last ~1 month in the mouse peripheral circulation (30).

NAC plays a protective role in mutagenesis and carcinogenesis through a variety of mechanisms investigated in experimental test systems (32,33), and is considered to be one of the most promising cancer chemopreventive agents (34). When given throughout duration of the experiment, oral NAC exerted differential protective effects on the monitored CS-induced cytogenetic alterations. This drug strongly inhibited the formation of CS-induced MN PAM, to such an extent that their frequency was not significantly different from background levels both during the period of exposure to CS and after discontinuation of exposure. However, in agreement with our previous study in rats (14), the protective effect was less evident towards formation of BN PAM, being statistically significant at three times only. It is well established that CS is clastogenic and can affect the mitotic spindle in rodent PAM (7,10,14–16). The present data suggest that distinctive CS components are responsible for the clastogenic and aneugenic properties of this complex mixture, NAC being more effective towards clastogens than towards aneugens.

NAC had differential effects on the formation of MN PCE in bone marrow and on the frequency of MN NCE in peripheral blood. In fact, administration of this thiol decreased the induction of MN PCE by CS, but not to a significant extent, which agrees with the conclusions drawn in a previous study evaluating modulation by NAC of MN PCE induced in the bone marrow of rats exposed to CS itself (14) and of mice treated with a polycyclic aromatic hydrocarbon (18). This lack of effect is consistent with the finding that, in mice treated i.p. with NAC, GSH stores were significantly increased in various tissues but were surprisingly depleted in bone marrow cells (35). In contrast, the frequency of MN NCE in CS-exposed mice receiving NAC was significantly lower than that observed in sham-exposed mice. Again, this agrees with the conclusions of previous studies evaluating modulation by NAC of MN erythrocytes in the peripheral blood of mice treated with doxorubicin (19,20) or urethane (21). Micronucleation occurs during erythropoiesis, and MN PCE migrate from bone marrow to the peripheral blood, where within 12–24 h they mature into circulating MN NCE (30). Alternatively, PCE mature into NCE when they still reside in the bone marrow (36). The greater inhibition exerted by NAC towards MN NCE in peripheral blood than towards MN PCE in bone marrow is difficult to interpret. It may tentatively be ascribed to the fact that inhibition by NAC, although it was not statistically significant in bone marrow cells, became manifest in the peripheral blood due to an accumulation of protective effects.
NAC enhances GSH stores in circulating erythrocytes, as shown in rats treated i.p. with the drug (37). Moreover, in comparison with blood, bone marrow contains more erythrocytes harboring micronuclei with whole chromosomes, which suggests that there will be more CS-induced MN cells with chromosome fragments in blood than in bone marrow. As NAC suppressively decreases clastogenic effects, its impact will be higher in blood than in bone marrow. A further tentative interpretation is that, although inactive in bone marrow, NAC may inhibit the formation of MN PCE in the spleen, which is an alternative erythropoietic organ in adult mammals (36,38).

When given after discontinuation of exposure to CS, in order to simulate the situation in ex-smokers, NAC did not modulate any of the monitored cytogenetic changes produced by exposure to CS. This lack of activity was easily predictable, since the cytogenetic alterations, once formed, are irreversible and persist until the affected cells are viable. Unless some agent may be able to impair the viability of cells carrying cytogenetic alterations, chemopreventive agents are expected to have beneficial effects in ex-smokers by modulating reversible mechanisms of the carcinogenesis process. Interestingly, NAC was found to normalize the cellularity of bronchoalveolar lavage, which 11 weeks after withdrawal of exposure to CS was still altered, with an almost 10-fold increase of PMN and a parallel decrease of PAM. This protective effect was significant both when NAC was administered throughout duration of the experiment and when it was administered after discontinuation of exposure to CS. The accumulation of polymorphonuclear leukocytes has also been reported to occur in smoking humans (39), and is considered to be a useful parameter for evaluating the pulmonary inflammatory response (40). Once these cells accumulate in the lung, it is possible that they may actually interfere with PAM functions (39). In a previous study in rats, oral NAC significantly decreased the stimulation of MN PCE consequent to exposure to CS, and attenuated the CS-induced emphysema and multiple hyperplastic and metaphlastic changes of bronchial and bronchiolar epithelia (14). This protective effect of NAC may tentatively be related to the proposed role of this thiol in modulating the production and release of cytokines (41), and in defending bronchoalveolar lavage cells from the toxic products, such as reactive oxygen species, generated during phagocytosis (42).

In conclusion, the present study provides a detailed analysis of the differential induction and persistence of cytogenetic alterations in PM, bone marrow PCE and circulating NCE of CS-exposed mice. The thiol NAC exerted protective effects on most of the monitored parameters when administered throughout duration of the experiment. When given after discontinuation of exposure to CS, NAC failed to affect the cytogenetic alterations but was successful in normalizing the bronchoalveolar lavage cellularity altered by the previous exposure to CS.

Acknowledgements
This study was supported by the Bulgarian Ministry of Education and Science and by the Associazione Italiana per la Ricerca sul Cancro (AIRC).

References


Received March 11, 1999; revised April 26, 1999; accepted April 27, 1999