Genetic Characterization of Diethylnitrosamine-induced Purple Adenine (ad-3) Mutants in Neurospora crassa

H. V. Malling and F. J. de Serres

SUMMARY

The mutagenic effect of diethylnitrosamine was studied in Neurospora crassa. The metabolic activation required to make diethylnitrosamine carcinogenic in vivo was mimicked in vitro with one of Udenfriend's hydroxylation mixtures (a mixture consisting of ascorbic acid, Fe++, and EDTA); only under these conditions was the compound found to be mutagenic. Mutants were induced in a genetically marked 2-component heterokaryon and recovered as purple colonies among the white, nonmutant background colonies. In this system, purple colonies result from two types of genetic damage in the adenine-3 region: point mutations at ad-3A and ad-3B loci and multilocus deletions covering one or both loci simultaneously. Genetic analysis showed that 99% of the diethylnitrosamine-induced ad-3 mutants were point mutations. Mutants in the ad-3B locus show allelic complementation, whereas those at the ad-3A locus do not. Among the diethylnitrosamine-induced ad-3B mutants, 65.5% had nonpolarized complementation patterns, 4.5% had polarized complementation patterns, and 29.9% were noncomplementing. Our previous studies have shown that mutants with nonpolarized patterns result from base-pair substitutions and probably produce a complete polypeptide chain with only a single erroneous amino acid substitution. Thus, our data on diethylnitrosamine support our hypothesis (Ann. N. Y. Acad. Sci., 163: 788–800, 1969) that potent carcinogenic activity is associated with gene products that have altered functions rather than with gene products that have no function.

INTRODUCTION

Diethylnitrosamine is known to be a potent carcinogen in many animal species (1, 12, 30, 33, 34), including primates (14). Diethylnitrosamine is metabolized by enzymes in many different organs to a strong alkylating agent (19). All suggestions (7, 8, 16, 18) about the active metabolic product involve an initial enzymatic oxidative dealkylation of nitrosamines.

In addition, diethylnitrosamine is mutagenic in Drosophila (9, 28). It is not mutagenic in Arabidopsis thaliana, but the closely related carcinogen DNM is (39). Most nitrosamines tested have been nonmutagenic in Escherichia coli (11, 29), Serratia (11), and yeast (27) and require metabolic activation in the host-mediated assay to become mutagenic in bacteria (10). DMN and diethylnitrosamine have induced reverse mutations in Neurospora crassa (20), but only when treatment has been performed in Udenfriend's hydroxylation mixture under O2 aeration (38).

The spectra of genetic alterations induced by 2 methylating compounds, MMS and MNNG, have been studied in N. crassa (25, 26). MMS is a weak carcinogen as compared to DMN (37) or MNNG (35). Comparison of the spectra of genetic alterations induced by MMS and MNNG has led to a working hypothesis (25) that carcinogenic activity of nitrosamines and nitrosamides is associated with the induction of a particular type of genetic alteration, those that give rise to gene products with altered function.

The mutagenic activity of diethylnitrosamine has been studied previously (20) in a reverse-mutation system, which did not give any quantitative indication of the types of genetic alteration induced. The present studies utilized the ad-3 test system in a 2-component heterokaryon of N. crassa developed by de Serres and coworkers (6, 40). With this system, the induction of recessive-lethal mutations can be studied at 2 closely linked specific loci, ad-3A and ad-3B. These mutations can be due either to a point mutation in 1 of the loci or a multilocus (or chromosome) deletion covering 1 or both of these loci in the ad-3 region. The complementation patterns of the ad-3B mutants can be determined and, by comparing the complementation pattern of diethylnitrosamine-induced ad-3B mutants with those of ad-3B mutants in which genetic alterations have been analyzed, tentative conclusions can be reached about the genetic alterations induced by diethylnitrosamine in Neurospora. This study has shown that 99% of the diethylnitrosamine-induced mutations are point mutations and that a high percentage of these point mutations specify proteins with altered function.

MATERIALS AND METHODS

Strains. All purple (ad-3) mutants were isolated by the direct method (5) after diethylnitrosamine treatment of a genetically marked 2-component heterokaryon of N. crassa

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2 The abbreviations used are: DMN, dimethylnitrosamine; MMS, methyl methanesulfonate; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine.
Diethylnitrosamine. Conidia from the 2-component heterokaryon contain an average of 2 nuclei. The conidia can either be homokaryotic for 1 or the other type of nucleus or they can contain 1 nucleus of each type. The fraction of each of these 3 types of conidia produced by a heterokaryon can be determined by plating on differentially supplemented media. If 1 nucleus in a heterokaryotic conidium is inactivated, the conidium is functionally homokaryotic. If mutagenic treatment causes inactivation of nuclei, the heterokaryotic fraction of the conidia is the most sensitive and is inactivated at a faster rate than the 2 homokaryotic fractions. In contrast, if mutagenic treatment causes cytoplasmic inactivation, all fractions of conidia are inactivated to the same degree.

The inactivation of the different fractions of conidia was measured for various lengths of time in Udenfriend’s hydroxylation mixture with and without diethylnitrosamine, with N₂ or O₂ bubbling through the reaction mixture. The inactivation curves are given in Chart 1. They show no difference between the inactivation of the heterokaryotic fraction and of the total population of conidia. Thus, none of the treatments resulted in nuclear inactivation. Diethylnitrosamine is much more toxic under N₂ bubbling than under O₂ bubbling, which is in contrast to the earlier results by Malling (20) for an ad-3 mutant of N. crassa, where
the toxicity of diethylnitrosamine was the same with both \(N_2\) and \(O_2\). At present we can give no rational explanation for this discrepancy. Diethylnitrosamine is mutagenic only in the mixture with \(O_2\). These data show, then, that the mechanism of inactivation with diethylnitrosamine and the mechanism of mutation induction are apparently unrelated.

Mutagenicity and Mutagenic Specificity of Diethylnitrosamine. The treatment of Neurospora conidia with diethylnitrosamine dissolved in Udenfriend's hydroxylation mixture and under \(O_2\) aeration was the only treatment that induced any significant increase in the forward-mutation frequency (Table 1). This agrees with the earlier observation (20) that diethylnitrosamine must be activated through hydroxylation to become mutagenic and that it is mutagenic only under those conditions that mimic the metabolic activation required for carcinogenic activity. Thus we have demonstrated a correlation between mutagenic and carcinogenic activity for diethylnitrosamine.

For determining whether there is a correlation between carcinogenic activity and the production of a particular type of genetic alteration, the spectrum of genetic alterations was determined by complementation tests and by dikaryon and trikaryon tests. These data show that only 1 mutant was classified as a multilocus deletion (\(ad-3^{IR}\)) in the \(ad-3\) region (Table 2); 99% of the diethylnitrosamine-induced \(ad-3\) mutations are point mutations. Of the point mutations, 26.8% of the mutants were of genotype \(ad-3A\) and 73.2% were of genotype \(ad-3B\). \(ad-3A\) mutants do not show interallelic complementation, but \(ad-3B\) mutants do, and their complementation responses can be grouped in 3 main classes: complementing mutants with nonpolarized, those with polarized complementation patterns, and noncomplementing mutants (3). Of the \(ad-3B\) mutants induced by hydroxylated diethylnitrosamine, 65.6% had nonpolarized complementation patterns, 4.5% had polarized complementation patterns, and 29.9% were noncomplementing.

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (hr)</th>
<th>Survival (%)</th>
<th>No. of (ad-3) mutations</th>
<th>Frequency of (ad-3) mutations/10⁶ survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>100</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Control + (O_2)</td>
<td>2</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control + (N_2)</td>
<td>2</td>
<td>107</td>
<td>5</td>
<td>1.0</td>
</tr>
<tr>
<td>Diethylnitrosamine + (O_2)</td>
<td>2</td>
<td>89</td>
<td>104</td>
<td>15.3</td>
</tr>
<tr>
<td>Diethylnitrosamine + (N_2)</td>
<td>2</td>
<td>86</td>
<td>1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

### Table 2

Percentages of different types of \(ad-3\) mutations induced by diethylnitrosamine + \(O_2\) in heterokaryotic conidia of \(N.\ crassa\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of purple colonies</th>
<th>(ad-3^{AR})</th>
<th>(ad-3^{BR})</th>
<th>(ad-3^{IR})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovered</td>
<td>Total analyzed</td>
<td>Too leaky for analysis</td>
<td>NP</td>
</tr>
<tr>
<td>Control(b)</td>
<td>7</td>
<td>93</td>
<td>2</td>
<td>(3)</td>
</tr>
<tr>
<td>Diethylnitrosamine + (O_2)</td>
<td>104</td>
<td>93</td>
<td>2</td>
<td>26.8 (25)</td>
</tr>
</tbody>
</table>

\(a\) NP, nonpolarized complementation pattern; P, polarized complementation pattern; NC, noncomplementing mutant.

\(b\) Includes the untreated control and the 2 treated controls.

\(c\) The number in parentheses indicates the total number of mutants in that class.
DISCUSSION

The present experiments with diethylnitrosamine show a correlation between carcinogenic and mutagenic activity. Diethylnitrosamine is not mutagenic per se but is only mutagenic with an in vitro treatment that mimics the in vivo metabolic activation that results in carcinogenic activity.

There is also a correlation between carcinogenic activity and the production of a particular type of genetic alteration. Genetic analysis of the diethylnitrosamine-induced ad-3 mutants shows that 99% are due to point mutations (alteration of the genes) and only 1% to multilocus deletions (physical removal of the gene from the chromosome by chromosome breakage). Genetic analysis of the point mutations to obtain a presumptive identification of the genetic alterations at the molecular level was made by tests for allelic complementation among the ad-3B mutants. Our previous studies (22–24) have shown a correlation between complementation pattern and genetic alteration at the molecular level. These data have led to the conclusion that mutants with nonpolarized complementation patterns are due mainly to base-pair substitutions, whereas mutants with polarized complementation patterns and noncomplementing mutants can arise from many types of genetic alteration. We also concluded that mutants with nonpolarized complementation patterns produce a complete protein with only a single erroneous amino acid substitution, whereas mutants with polarized complementation patterns and noncomplementing mutants produce nonfunctional proteins or polypeptide fragments. Mutants with nonpolarized patterns, which specify a complete polypeptide chain with altered function, are the only mutants that can be leaky (can grow on minimal medium without adenine); mutants with polarized patterns or noncomplemented mutants are never leaky (3).

The complementation spectrum among mutants induced by different mutagens varies considerably (3, 25), as illustrated in Table 3. The frequency of nonpolarized complementation patterns among MNNG- and diethylnitrosamine-induced ad-3B mutants is significantly higher than the frequency among MMS-induced ad-3B mutants (25), showing a correlation between carcinogenic activity and the production of a particular type of genetic alteration (those base-pair transitions that produce missense mutations).

MNNG, diethylnitrosamine, and MMS all result in 7-alkylated guanine (15, 17, 36, 37). MNNG and diethylnitrosamine result in a higher proportion of O-6-alkylated guanine than MMS. It seems likely that the differences in the complementation patterns could be explained on the basis of the alkylated minor component of the DNA and that this minor component could result in mutants with nonpolarized complementation patterns. If we assume that O-6-methyl-guanine induces base-pair transitions from GC to AT and plays the major role in the induction of mutations, then the complementation spectrum of MNNG-induced ad-3B mutants should be similar to the complementation spectrum of hydroxylamine-induced ad-3B mutants, which mainly have AT at the mutation site (21); however, this is not the case (Table 3). For an explanation of our data, we must assume that minor alkylation products of DNA, not yet identified, play a major role in the induction of mutation.

In summary, the data from the genetic tests on diethylnitrosamine-induced ad-3B mutants show a correlation between potent carcinogenic activity and the production of a particular type of genetic alteration. Essentially all of the diethylnitrosamine-induced mutants result from alterations of DNA that produce point mutations, many of which specify proteins with altered function. Thus the data from the present experiments support our working hypothesis (25) that potent carcinogenic activity is associated with the production of gene products with altered function rather than with gene products with no function.

ACKNOWLEDGMENTS

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REFERENCES

Diethylnitrosamine as a Mutagen


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