An adaptive response to alkylating agents in *Aspergillus nidulans*

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Summary. A simple method is described for demonstrating adaptation to alkylation damage in *Aspergillus nidulans*. One wild type, two MNNG-sensitive, and one MNNG-resistant strain all showed improvement in colony growth when challenged with MNNG following appropriate inducing pretreatments. Other alkylating agents (MMS, EMS) could also adapt mycelium to later MNNG challenge, while 4NQO and UV could not. The inducible effect was not transmissible through conidia. A standard reversion assay based upon *methG* proved impractical for studying mutation frequencies during alkylation treatments owing to variations in MNNG resistance amongst revertants.

Key words: *Aspergillus nidulans* – DNA repair – Induction – Alkylating agents

Introduction

When exposed to DNA damaging agents, cells from a range of organisms exhibit induction of repair gene expression to produce the appropriate repair enzymes. For example, when *E. coli* is exposed to low concentrations of alkylating agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) or methyl methane sulphate (MMS), cells become adapted to subsequent challenge doses of these compounds. In this so-called "adaptive response", DNA glycosylase and DNA alkyl-transferase repair proteins are induced, and they subsequently account for the improved responses to both the lethal and mutagenic effects of ensuing alkylating agent treatment (see Sedgwick 1986 for review). An analogous adaptive response involving the induction of repair enzymes has also been observed in some higher

mammalian systems (e.g., Samson and Schwartz 1980; Laval and Laval 1984) and in higher plants (reviewed by Soyfer 1987). In lower eukaryotes, however, no such adaptive response has been reported, and is apparently absent from the best characterized organism, *Saccharomyces cerevisiae* (Maga and McEntee 1985; Polakowska et al. 1986). Because of the advantages that lower eukaryotes offer with respect to rapid growth and ease of genetic analysis, we have turned our attention to another genetically well-characterized organism, *Aspergillus nidulans*. While a number of mutants of *A. nidulans* show increased sensitivity to alkylating agents, none of those so far studied appear to be defective in either DNA glycosylase or alkyl-transferase activities (Swirski et al. 1988). By analogy with *E. coli*, one way to isolate such defective mutants of this filamentous fungus would be to establish conditions for the adaptation of *A. nidulans* to alkylating agent damage (Jeggo et al. 1977) and then to screen sensitive mutants for the absence of this response (Jeggo 1979).

We describe here a simple method for inducing increased resistance to alkylating agents in MNNG-sensitive, wild-type and MNNG-resistant strains of *A. nidulans*.

Materials and methods

Strains. Wild-type strain L20 (*wA3 pabaA1*), MNNG-sensitive strains L451 (*wA3 pabaA1 sagA1*) and L453 (*wA3 pabaA1 sagC3*), along with the MNNG-resistant strain L601 (*wA3 pabaA1 rag1*) were used (Swirski et al. 1988; Hooley et al. 1988). Strain L601 is RAG1 of Hooley et al. (1988). Strain O110 (*biA1 methG*) was used to measure mutation/reversion frequencies of *methG* (Lilly 1965).

Media. Growth media were as described by Pontecorvo et al. (1953) and Clutterbuck (1974). MNNG from a freshly made stock of 400 $\mu\text{g} \cdot \text{ml}^{-1}$ in ethanol was added to cool agar. 4-

Nitroquinoline-1-oxide (4NQO) was kept as a stock solution at $1 \text{ mg} \cdot \text{ml}^{-1}$ in ethanol and added directly to the cool agar. Ethyl methane sulphonate (EMS) or MMS were added to the media together with $50 \mu\text{l}$ absolute ethanol per 20 ml media to help solubilize these agents.

Sensitivities of strains to MNNG were assessed by comparing colony extension rates with those of MNNG-free controls.

All experiments were performed at 37°C .

Induction treatment. Growth conditions were chosen which allowed maximum vegetative growth before the onset of sporulation. Conidia were spread across plates of CM alone or CM containing MNNG (0.25 or $1.0 \mu\text{g} \cdot \text{ml}^{-1}$) and incubated for 20 h . After this period of growth, plates were normally covered with vegetative mycelial lawns having aerial hyphae. The plates were then stored at 6°C in darkness for 6 h while challenge plates were prepared. Subsequent tests showed that a 20 h adaptation period produced the maximum effect with respect to subsequent growth in the presence of MNNG. A number of shorter exposure periods were also investigated, with 3 h being the minimum length of time which produced an observable effect.

Challenge treatment. Mycelial plugs, 6 mm in diameter, were taken from pretreated induction plates and inoculated face down onto CM plates containing various concentrations of MNNG. Four to six replicate inocula were made for each strain/induction/challenge treatment combination. Three random diameters were measured for each colony after 20 h growth and, taking into account the original inoculum size, growth was expressed as the radial extension rate over this period.

Tests of stability. The stability of inducible responses was tested in two ways. Firstly, L20 conidia from 2-day-old plates on CM alone or CM containing MNNG ($1.0 \mu\text{g} \cdot \text{ml}^{-1}$) were point inoculated onto CM containing varying doses of MNNG. Colony diameters were measured after 20 h growth at 37°C . Secondly, conidial subcultures from the same original 2-day-old plates were point inoculated onto plates of CM alone and allowed to grow for 5 days. Conidia taken from the edge of the plate were then tested.

Biomass measurements. An analogous method was used to study inducible effects in liquid culture. Conidial suspensions of L20 (approximately 10^6 spores) were incubated (shaken, 37°C , 200 rpm) for 5.5 h (to allow spore germination) in 7.5 ml of MM (paba) in Universal tubes. MNNG $1 \mu\text{g} \cdot \text{ml}^{-1}$ (in ethanol) was added to three tubes, and the same volume of ethanol alone added to three control tubes. Following an incubation of 20 h , these tubes were vibro-mixed, and the contents of each added individually to 200-ml flasks containing MNNG ($2 \mu\text{g} \cdot \text{ml}^{-1}$). These flasks were incubated for a further 24 h . At the end of this period, the pellets were washed in a saturated solution of sodium thiosulphate, filtered and dried to a constant weight.

Cross-induction by other DNA damaging agents. Conidia were spread on CM plates, incubated for 20 h at 37°C , and the plates were then stored at 6°C until the irradiation treatment. The plates were irradiated with UV at a dose rate of $1 \text{ J} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ within a dose range of $0\text{--}1,200 \text{ J} \cdot \text{m}^{-2}$ ($0\text{--}300 \text{ J} \cdot \text{m}^{-2}$ at $30 \text{ J} \cdot \text{m}^{-2}$ intervals, $300\text{--}1,200 \text{ J} \cdot \text{m}^{-2}$ at $150 \text{ J} \cdot \text{m}^{-2}$ intervals). Following irradiation, the plates were kept in the dark. The challenge treatment was then as described for MNNG.

Other chemical mutagens (MMS, EMS and 4NQO) were added to CM plates, which were then inoculated with L20

conidia. The incubation times and challenge treatments were then as for MNNG.

MMS was used at concentrations up to $100 \mu\text{g} \cdot \text{ml}^{-1}$, EMS up to $1,500 \mu\text{g} \cdot \text{ml}^{-1}$ and 4NQO up to $1.5 \mu\text{g} \cdot \text{ml}^{-1}$.

Mutagenesis. We attempted to assess the degree of protection against mutation as a consequence of pretreatment by using the reversion of *methG* as described by Lilly (1965). This system is widely used because of its sensitivity: *methG* revertants of three different distinguishable types (A, B, and C) arise as a result of mutations at different suppressor loci. The frequency of reversion of *methG* was scored in spores from challenge plates (MNNG $2 \mu\text{g} \cdot \text{ml}^{-1}$) incubated for 48 h after inoculation with plugs of 0110 mycelium either taken from MNNG-free control plates or from mycelium previously exposed to $1 \mu\text{g} \cdot \text{ml}^{-1}$ MNNG for 20 h . The prolonged incubation period (48 h) was necessary to allow for sporulation. Spores were pre-germinated and washed before plating on selective media (MM + bi). Numbers and types of revertants were scored after $3\text{--}4$ days, at dilutions which gave $1\text{--}20$ colonies per plate (Scott et al. 1973). The frequency of reversion to methionine prototrophy was then expressed as revertants per viable spore (assessed as colony formation on non-selective, methionine-supplemented medium, compared with total haemocytometer counts).

Results

Adaptive responses to MNNG

Pretreatment of wild-type L20 mycelium with $1 \mu\text{g} \cdot \text{ml}^{-1}$ MNNG for 20 h considerably improved growth responses upon subsequent challenge with MNNG. An MNNG-resistant mutant L601 *rag1* also showed this adaptive response, but increasing the pretreatment doses to 2 or $4 \mu\text{g} \cdot \text{ml}^{-1}$ MNNG failed to induce growth further upon subsequent MNNG challenge.

Two MNNG-sensitive strains (L451 *sagA1* and L453 *sagC3*) failed to show any inducible response after a $1 \mu\text{g} \cdot \text{ml}^{-1}$ MNNG pretreatment; indeed, an additional toxicity was evident (Fig. 1). However, when pretreatment doses were lowered to $0.25 \mu\text{g} \cdot \text{ml}^{-1}$ MNNG, both wild-type and sensitive mutants showed clear inducible improvements in survival rate following a challenge dose (Fig. 2). In all the strains tested, a 20 h induction period was found to produce maximal effects, although a small degree of adaptation could be detected after induction periods as short as 3 h . For periods shorter than this, no adaptation could be detected. Attempts to observe adaptation effects with germinating conidia were limited by the amount of exposure which could be given before mycelial growth became too profuse. Consequently, we found the plate test gave the clearest indication of the effects.

In addition to improved colony growth rates, "induced" cultures on plates appeared denser and showed less brown pigmentation than "control" cultures. In order to assess the effects of adaptation on biomass

Fig. 1A-C. Hyphal (colony radial) extension rates of three strains pre-treated with MNNG ($1 \mu\text{g} \cdot \text{ml}^{-1}$) and challenged with increasing doses of MNNG. A L20 wild-type control: pretreated ($\bullet$); untreated control ($\circ$). B L451 *sagA1* MNNG-sensitive mutant: pretreated ($\bullet$); untreated control ($\circ$). C L601 *rag1* MNNG-resistant mutant: pretreated ($\bullet$); untreated control ($\circ$) (means, ± 2 standard errors)

rather than on radial growth rates, dry weight measurements were made of L20 flask cultures with and without pre-induction. These treatments revealed clear differences in biomass on challenge with MNNG. The mean dry weight per 200-ml flask was 38.7 mg for control mycelium (41.0, 40.0, 35.0 mg) and 110.3 mg for induced mycelium (144.0, 130.0, 47.0 mg), when grown for 24 h in MM + $2 \mu\text{g} \cdot \text{ml}^{-1}$ MNNG. It is clear, therefore, that the adaptive response can be induced in both plate-grown and liquid-grown cultures.

Cross induction with other mutagens

Pretreatments with 4NQO or a range of UV doses failed to induce increased resistance to MNNG on subsequent challenge with MNNG. At most doses, pretreatment in fact caused a reduction in the growth of colonies challenged with MNNG (Fig. 3, for example).

Fig. 2A, B. Hyphal (colony radial) extension rates of two strains pre-treated with MNNG ($0.25 \mu\text{g} \cdot \text{ml}^{-1}$) and challenged with increasing doses of MNNG. A L20 wild-type control: pretreated ($\bullet$); untreated control ($\circ$). B L451 *sagA1* MNNG-sensitive mutant: pretreated ($\bullet$); untreated control ($\circ$) (means, ± 2 standard errors)

In contrast, both MMS and EMS at appropriate concentrations were capable of inducing improved colony growth rates upon subsequent challenge with MNNG. Pretreatment with MMS at less than $10 \mu\text{g} \cdot \text{ml}^{-1}$ failed to induce increased resistance, while above $50 \mu\text{g} \cdot \text{ml}^{-1}$, additional toxicity was observed (Fig. 4). Induction by EMS pretreatment was less marked and complicated by the fact that low concentrations of this compound improved colony growth rates upon subculture to MNNG-free CM (not shown).

Stability of the inducible response

The inducible resistance to MNNG challenge was not transmissible through conidia. Spores taken from "induced" and "control" L20 pretreatments showed no differences in colony growth rates or densities on subsequent challenge with a range of MNNG concentrations. Asexual spores did not show adaptation to further MNNG challenge.

However, when the viabilities of 0110 spores taken from 48-h-old MNNG ($2 \mu\text{g} \cdot \text{ml}^{-1}$) challenge plates

Fig. 3. Hyphal (colony radial) extension rates of a wild-type strain (L20) pre-treated with UV ($60 \text{ J} \cdot \text{m}^{-2}$) and challenged with increasing doses of MNNG: pretreated with UV (●); untreated control (○) (means, ± 2 standard errors)

Fig. 4. Hyphal (colony radial) extension rates of a wild-type strain (L20) pre-treated with MMS (25 or $100 \text{ μg} \cdot \text{ml}^{-1}$) and challenged with increasing doses of MNNG: untreated control (○); pretreated with $25 \text{ μg} \cdot \text{ml}^{-1}$ MMS (Δ); pretreated with $100 \text{ μg} \cdot \text{ml}^{-1}$ MMS (▲) (means, ± 2 standard errors)

Table 1. Spore viability and reversion to methionine prototrophy in control and induced cultures of strain 0110

Culture	Spore viability (%)		Apparent reversion frequency	Total number revertants screened	No. of revertant types ^b		
	I	II ^a			A	B	C
Control	42.5	29.4	1 in 5,049 spores	76	48	26	2
"Induced"	82.5	41.1	1 in 336 spores	154	128	17	9

^a 2 experiments

^b After Lilly (1965); distribution of revertant types are different between control and induced ($P < 0.001$; $\chi^2_2 = 40.61$)

were compared from two experiments, the pretreated cultures showed consistently higher viabilities (Table 1), as might be expected of material from which more DNA damage had been removed.

Mutagenesis

Using the *methG* reversion assay described in the Materials and methods, initial results indicated an increase in mutation frequency following pretreatment, and not a decrease. However, as the distribution of revertant types among pretreated and control cultures was rather different, a comparison was made of the MNNG sensitivities of the three revertant types (A, B and C) and parental strain 0110. It was revealed that the revertants differed in their responses. Three out of the three type A revertants tested were considerably more resistant to MNNG than was 0110. A single type C revertant was also more resistant, while both of the type B's tested were more sensitive to MNNG than the auxotrophic parent (data not shown). These different sensitivities to MNNG, based presumably upon mutation at specific

suppressor loci, were reflected in the different proportions of revertant types observed (Table 1). The increased resistance of type A and type C revertants to the killing effects of MNNG clearly means that such revertant types will be at a selective advantage during the adaptation period, resulting in a substantial distortion in the results. These data cannot, therefore, be meaningfully compared to the untreated control. This aspect of the work is considered further in the Discussion section.

Discussion

Pretreatment of the vegetative mycelium of *A. nidulans* with low doses of MNNG improves responses on subsequent challenge with higher doses of alkylating agents. Specific MNNG concentrations are required for pretreatment dosages, and sensitive mutants (L451 *sagA1* and L453 *sagC3*) can still show an adaptive response provided inducing doses are low enough. A resistant mutant (L601 *rag1*) also shows the inducible

response, which improves its MNNG resistance still further. These observations resemble those of Jeggo et al. 1977, who provided varying dose pretreatments to induce *E. coli* strains of differing alkylating agent sensitivity.

Clearly, the MNNG sensitivity of L451 and L453 cannot be accounted for by the failure of this inducible response. Likewise, the MNNG resistance of L601 cannot be the result of a constitutive expression of this normally inducible response.

The adaptive increase in MNNG resistance is not a long-term, stable change and is lost on passage through spores and upon further sub-culture. Pretreatments with both EMS and MMS induced improved responses to later challenge doses of MNNG, while both UV and 4NQO pretreatments were unable to elicit such a response. Although this may reflect our inability to find precise inducing treatments, it strongly suggests that the adaptive response is specific to alkylating agents. A similar pattern of response was found in the two mutant strains L451 and L453. An interesting feature of L453 *sagC3* was that following induction the resistance to low challenge doses of MNNG became almost that of wild-type strain L20.

The reversion of *methG* proved inappropriate as a measure of mutagenesis during and following adaptation – the variation in MNNG responses of the different revertant types preventing a meaningful interpretation of the data. The alkylating agent sensitivity of all amino acid auxotrophs in *Aspergillus* (Kafer 1987) makes such loci of dubious value in measuring mutation frequencies associated with alkylation damage, and we are currently attempting to develop a protocol to overcome this particular problem. We also believe it probable that the different sensitivities of the *methG* revertants account for the apparent interlocus specificity of EMS reported in *A. nidulans* (Alderson and Clark 1966), and that this is not a real effect. It is clear from our data, however, that despite the distortions caused by the different MNNG sensitivities, pretreatment does not result in a dramatic reduction in mutation frequency.

Maga and McEntee (1985) and Polakowska et al. (1986) have presented evidence for the absence of an adaptive response following treatment of *Saccharomyces cerevisiae* with MNNG, and have concluded that repair mechanisms in this lower eukaryote are quite different from those of *E. coli*. This behaviour can now be clearly seen as a feature of *S. cerevisiae* alone, and not as a property common to all lower eukaryotes. The adaptive response in *E. coli* is a result of the induction of both O⁶ alkylguanine DNA alkyltransferase and 3-methyladenine-DNA glycosylase II activities (for review Sekiguchi and Nakabeppu 1987). However, Swirski (1986) was unable to demonstrate changes in DNA glycosylase

or DNA alkyltransferase activities in *A. nidulans* following pretreatments with MNNG, although this may be the result of inappropriate induction/challenge conditions or poor in vitro assay sensitivity.

The nature of the adaptive response in *A. nidulans*, therefore, remains to be elucidated. Increased UV resistance in the fungus *Ustilago maydis* has been ascribed to an inducible DNA repair process (Lee and Yarranton 1982); the same appears to be true for *Saccharomyces cerevisiae* (e.g. Siede and Eckardt 1984; Rolfe 1985a, b) and *Neurospora crassa* (Baker 1983; Howard and Baker 1986). We are currently attempting to follow the kinetics of the removal of alkylation products from the DNA of induced and non-induced cultures of *A. nidulans* to determine whether the effects are mediated at this level. This work will be reported elsewhere.

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