Mutants of *Neurospora crassa* Resistant to 8-Azaguanine

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SUMMARY

Under conditions of limited-adenine supplementation, 8-azaguanine is a potent inhibitor of purine auxotrophic strains of *Neurospora crassa*. It is relatively ineffective in prototrophic strains. Spontaneous and u.v.-light-induced mutants, resistant to 8-azaguanine, were obtained in a strain carrying the *ad-2*, *ad-3A*, and *ad-3B* markers. The resistant mutants grew in the presence of 200 µg 8-azaguanine and 2.0 µg adenine sulphate/ml medium. Inhibition of the sensitive strain is virtually complete under these conditions. Higher adenine sulphate concentrations prevent inhibition of the sensitive strain by 8-azaguanine. Resistance of the new mutants to 8-azaguanine was demonstrated by growth-tube experiments and by plating efficiencies in the presence and in the absence of the inhibitor. Growth-tube experiments were also used to study the reversal of 8-azaguanine induced inhibition by adenine sulphate. The resistance markers in the five strains studied were assigned to linkage group III. The locus designation *aza-3* was given to the resistance marker in a selected mutant of spontaneous origin. Resistance to 8-azaguanine is stable in the presence and in the absence of the inhibitor. In heterokaryons, 8-azaguanine resistance is recessive.

INTRODUCTION

The purine analogue 8-azaguanine (azaguanine) is a growth inhibitor in micro-organisms and in mammalian cells. It is incorporated into RNA, and possibly to a much lesser extent into DNA (Balis, 1968; Harbers, Domagk & Muller, 1968; Roy-Burman, 1970). The biological effects of azaguanine are generally attributed to interference with protein synthesis caused by modification of RNA (Harbers *et al.* 1968; Mahadevan & Bhagwat, 1969). In addition to the formation of faulty RNA, the mechanism of action of azaguanine probably involves feedback inhibition of the production of normal guanylic acid (Balis, 1968). Conversion of the free base to azaguanic acid is common to both mechanisms (Balis, 1968). Effects of azaguanine on DNA and RNA synthesis have also been reported (Perevoschikova, 1969; Rode & Bayen, 1972).

Resistance to azaguanine has been reported in a variety of experimental organisms and is most frequently attributed to an alteration or loss of hypoxanthine-guanine phosphoribosyltransferase (HGPRT) activity (Balis, 1968; Brockman, Sparks, Hutchinson & Skipper, 1959; Harbers *et al.* 1968; Roy-Burman, 1970). Other reported mechanisms of resistance to azaguanine include specific loss of guanine phosphoribosyltransferase activity (Kalle & Gots, 1961), decreased cell permeability to the analogue (Harbers *et al.* 1968; Szybalski, Szybalska & Ragni, 1962), efficient deamination of azaguanine to the relatively inactive 8-azaxanthine (Harbers *et al.* 1968; Szybalski *et al.* 1962), and altered feedback inhibition of the purine biosynthetic pathway (Heslot, Nagy & Whitehead, 1966; Shiio & Ishii, 1971).

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Selection for azaguanine resistance in *Neurospora* was reported by Bedair & Fuerst (1961), but resistant mutants were not genetically characterized. Jha (1971) later described the induction of 8-azaadenine resistant mutants at two loci in *Neurospora*. Several of the mutants were reported to be cross-resistant to azaguanine.

The present investigation describes mutants of *Neurospora crassa* which are resistant to the effects of azaguanine. These mutants are being used in the development of a new system for the detection of forward mutations (Hoffmann & Malling, 1974).

METHODS

Strains. Mutants resistant to azaguanine were induced in a strain of *Neurospora crassa* (74-OR60-29A) with the following genetic markers: A, hist-2, ad-3A, ad-3B, nic-2, ad-2, inos. This strain, a strain (12-007-0073) with the A, ad-3B, al-2, cot, and pan-2 markers, and a strain carrying an mtr marker, were obtained from the culture collection of F. J. de Serres, National Institute of Environmental Health Sciences. Linkage studies were carried out with the alcoy multiple translocation stocks and follow-up testers developed by Perkins, Newmeyer, Taylor & Bennett (1969). These strains were obtained from the Fungal Genetics Stock Center, Humboldt State College, Arcata, California, U.S.A.

Media. The plating medium used for colonial growth was Westergaard's basal medium (Westergaard & Mitchell, 1947; de Serres & Malling, 1971) containing 0.75 % L-sorbose, 0.05 % D-glucose, and 0.05 % D-fructose. For uninhibited mycelial growth and conidiation, Westergaard's or Fries basal medium (Horowitz & Beadle, 1943; de Serres & Malling, 1971) containing 1 % D-glucose was used. When required, the following nutritive supplements were added to the medium: adenine sulphate (100 mg/l for full supplementation, 2 mg/l for limited adenine conditions), DL-histidine-HCl-H₂O (100 mg/l), inositol (30 mg/l), nicotinamide (10 mg/l), D-calcium pantothenate (10 mg/l), L-tryptophan (100 mg/l). The Difco Bacto-agar (2.0 %) used in solid media was washed three times in distilled water. Azaguanine was purchased from Schwarz-Mann Chemical Co. Stock solutions of 10 mg azaguanine/ml (6.58×10^{-2} M) were prepared by dissolving the azaguanine in a minimal volume of 1 N-NaOH and diluting to the final volume with distilled water. Various amounts of filter-sterilized azaguanine were added to the medium immediately before use.

Neurospora techniques. The procedures followed in handling the experimental organism have been described in detail by de Serres & Malling (1971).

Growth comparisons among strains and at different chemical concentrations were made in 20 × 150 mm growth tubes, as previously described (Hoffmann, Malling & Mitchell, 1973). Growth tubes were inoculated with single colonies isolated from plates. The point of furthest advance of the mycelial front was marked at 12 h intervals while the tubes were incubated horizontally at 30 °C. Measurements were made after the mycelial front reached the end of the tube. For plating-efficiency determinations, 200 conidia, determined by haemocytometer counts, were plated in Westergaard's plating medium under limited-adenine conditions. Colony formation in non-inhibitory medium was compared to that in medium containing 200 µg azaguanine/ml. Colony counts after 3 days of incubation at 30 °C were expressed as a percentage of the conidia plated.

All crosses were made in 20 × 150 mm test tubes on supports made from Whatman No. 1 filter paper. Ten millilitres of Westergaard's basal medium containing 0.2 % sucrose were dispensed per tube. The medium was supplemented for the nutritional requirements of the strains crossed. The crosses were incubated for 6 weeks at 25 °C. Preparation of ascospore suspensions was carried out by the procedure of F. J. de Serres (Hoffmann *et al.* 1973).

Before plating, the ascospores were heat shocked in 0.15 % purified agar for 50 min at 60 °C. Ascospores were plated in Westergaard's plating medium, supplemented for the two parent strains. After two to three days' incubation at 35 °C, colonies were transferred to slants of Fries conidiating medium. All further tests were made with dilute suspensions of conidia grown on this medium. Auxotrophic markers were scored after 3 days' incubation at 25 °C in Westergaard's liquid medium lacking only the nutrient being tested. Sensitivity or resistance to azaguanine was evaluated by a spot plate technique. A drop of conidial suspension was transferred on an applicator stick to one of eight spots on a Petri plate of supplemented plating medium containing 200 µg azaguanine and 2 µg adenine sulphate/ml. After 3 days' incubation at 30 °C, resistance and sensitivity were scored by comparative growth on the spots.

Selection of mutants. Mutants were induced with u.v. light. Ultraviolet irradiation was performed with a 15 W, No. G15-T8 G.E. germicidal lamp. A dose of 2000 erg/mm² was administered at the dose rate of 18.5 erg/mm²/s, as measured with a Jagger meter (Jagger, 1961). Conidial suspensions were counted in a haemocytometer and adjusted to the concentration of 2×10^6 conidia/ml. Samples (25 ml) were irradiated in 15 × 100 mm glass Petri plates. The conidial suspensions were stirred on a magnetic stirrer during irradiation. Irradiation and plating were carried out in a room illuminated with red light in order to prevent photo-reactivation. Mutants were selected in Westergaard's plating medium containing 200 mg azaguanine and 2 mg adenine sulphate/ml. Plates were incubated at 30 °C in the dark. Mutants that arose in the selection medium were made genetically homogenous by repeated single colony isolations from media containing azaguanine and limited adenine as above.

RESULTS

Inhibition and mutant selection

At 200 µg/ml, azaguanine was effective in inhibiting colony formation when 200 prototrophic conidia were plated in 20 ml medium. When 10^6 conidia were plated in 20 ml medium the background growth was heavy, particularly after 4 or 5 days of incubation. Inhibition of growth remained incomplete at azaguanine concentrations up to 500 µg/ml. Higher concentrations were not tested.

When adenine auxotrophic conidia were plated in fully supplemented Westergaard's plating medium containing azaguanine, no inhibition was observed. Azaguanine becomes an effective inhibitor, however, at low adenine sulphate concentrations. Adenine sulphate concentrations as low as 0.5 µg/ml supported colony formation in Westergaard's plating medium. Inhibition of growth by 200 µg azaguanine/ml was virtually complete at adenine sulphate concentrations from 0.5 to 2.0 µg/ml. At higher adenine sulphate concentrations, inhibition by azaguanine was reversed.

Since azaguanine is a very effective inhibitor in an adenine auxotrophic strain under limited-adenine conditions, azaguanine resistant mutants were selected in Westergaard's plating medium containing 200 µg azaguanine and 2.0 µg adenine sulphate/ml. The level of background growth under these conditions is very low.

Azaguanine resistant mutants were obtained after u.v. irradiation at a dose of 2000 erg/mm² (78 % survival). Five strains which formed colonies of normal morphology in the presence and absence of azaguanine were selected for further study. These strains will be referred to as CI-8AG no. 1, no. 2, no. 4, no. 6, and no. 10. Strains CI-8AG no. 1, no. 2, no. 4 and no. 10 were derived from mutants induced by u.v. light, while CI-8AG no. 6 arose as a spontaneous mutant. The colonies produced by the five azaguanine resistant strains in Westergaard's

Fig. 1. The effect of azaguanine on the sensitive strain and a resistant strain in growth tubes under limited-adenine conditions. (a) The sensitive strain, 74-OR60-29A; (b) CI-8AG no. 6. Azaguanine concentrations ($\mu\text{g/ml}$): $\circ$ — $\circ$, 0; $\bullet$ — $\bullet$, 25; $\triangle$ — $\triangle$, 50; $\blacktriangle$ — $\blacktriangle$, 100; $\square$ — $\square$, 200.

Fig. 2. Reversal of azaguanine-induced inhibition by adenine sulphate in the sensitive strain 74-OR60-29A. (a) Absence of azaguanine; (b) presence of azaguanine (200 $\mu\text{g/ml}$). Adenine sulphate concentrations ($\mu\text{g/ml}$): $\circ$ — $\circ$, 2; $\bullet$ — $\bullet$, 4; $\triangle$ — $\triangle$, 8; $\blacktriangle$ — $\blacktriangle$, 16; $\square$ — $\square$, 32; $\blacksquare$ — $\blacksquare$, 100.

plating medium without azaguanine were somewhat larger than those produced by the sensitive strain. The five CI-8AG strains grew and conidiated well on Fries conidiating medium.

Growth studies

Growth-tube experiments were used to demonstrate the resistance of the CI-8AG strains and to study the effect of adenine sulphate on inhibition by azaguanine. The advance of a mycelial front at several azaguanine concentrations was measured in Westergaard's conidiating medium at the adenine sulphate concentration of 2.0 $\mu\text{g/ml}$. Results for the sensitive strain and for strain CI-8AG no. 6 are presented in Fig. 1, where each plotted point represents the mean of four growth tubes. Results for the four other resistant strains are the same as those for strain CI-8AG no. 6. Fig. 2 illustrates the effect of adenine sulphate on inhibition of the sensitive strain by azaguanine. The growth-tube data illustrate the resistance of the CI-8AG strains to azaguanine (Fig. 1) and the reversal by adenine sulphate of the effects of azaguanine in the sensitive strain (Fig. 2).

In addition to the growth-tube experiments, resistance was evaluated by plating efficiency determinations. The data are presented in Table 1. Each value represents the mean of three

Table 1. *Plating efficiencies in the presence and absence of azaguanine under limited-adenine conditions*

Strain	Plating efficiency (%)	
	No inhibitor	Azaguanine (200 µg/ml)
Sensitive (74-OR60-29A)	68	0
CI-8AG no. 1	83	70
CI-8AG no. 2	68	84
CI-8AG no. 4	86	76
CI-8AG no. 6	74	71
CI-8AG no. 10	86	78

plates. The plating efficiencies indicate that the CI-8AG strains are resistant to azaguanine in plating medium under limited-adenine conditions.

The growth-tube and plating-efficiency experiments support one another in indicating that the new mutants are resistant to azaguanine under the specified conditions. The characteristics of the CI-8AG strains remain unchanged after many transfers, and resistance is stable in cultures grown either in the presence or in the absence of azaguanine.

Cross-resistance evaluations were made by surface plating conidia on limited-adenine medium containing various concentrations of the inhibitor in question. Both the sensitive strain and the CI-8AG strains are sensitive to the effects of the pyrimidine analogues, 5-fluorouracil and 5-fluorodeoxyuridine (FdUrd) at concentrations which do not inhibit FdUrd-resistant strains (Hoffmann *et al.* 1973). The amino acid analogue, DL-4-methyltryptophan, at a concentration of 110 µg/ml, is inhibitory to the sensitive strain and to CI-8AG no. 6. Other CI-8AG strains were not tested. A methyltryptophan-resistant (mtr) mutant, which was used as a positive control, formed colonies in the presence of the inhibitor. At 25 µg/ml, DL-4-methyltryptophan was partially inhibitory to both the sensitive strain and CI-8AG no. 6. At concentrations as high as 200 µg/ml, the adenine analogue 8-aza-adenine was not sufficiently inhibitory to permit cross-resistance evaluations. Inhibition by the hypoxanthine analogue 6-mercaptopurine at a concentration of 200 µg/ml was incomplete. While CI-8AG no. 6 produced colonies of good morphology, growth of the sensitive strain was more restricted. Strain CI-8AG no. 6 is apparently cross-resistant to 6-mercaptopurine.

The ability of the CI-8AG strains to use hypoxanthine as their sole purine supply was checked in Westergaard's plating medium. Even at concentrations lower than 0.5 µg/ml, colonies were formed.

Genetic studies of the azaguanine resistant mutants

Perkins' (Perkins *et al.* 1969) alcoy tester strains were used in the determination of the linkage groups of the resistance markers. Only adenine-auxotrophic progeny were scored for sensitivity or resistance because azaguanine resistance is not clearly expressed in prototrophic strains. Results from the alcoy cross indicated that the resistance marker in each of the five strains is linked to the ylo-1 locus in the tester strain. This result indicated that the azaguanine-resistance gene is located in either linkage group III or linkage group VI because the tester strain includes a translocation involving these two linkage groups.

The standard follow-up tester strain recommended by Perkins (Perkins *et al.* 1969) was used to distinguish between linkage groups III and VI. This strain is marked by a tryptophan requirement (tryp-1) in linkage group III and the yellow locus (ylo-1) in linkage group VI.

As in the previous series of crosses, only adenine auxotrophic progeny were tested for resistance. Results from this cross indicated linkage of the resistance marker in all five strains to *tryp-1* in linkage group III.

Jha (1971) described 8-azaadenine resistant mutants, some of which are cross-resistant to the effects of azaguanine. Jha's mutants were mapped to two loci, designated *aza-1* and *aza-2*, in linkage group I. The assignment of the resistance markers in the CI-8AG strains to linkage group III indicates that these genes are not the same as those reported by Jha.

The new azaguanine resistant mutant in strain CI-8AG no. 6 was given the locus designation *aza-3*. Since the possibility of more than one azaguanine-resistance gene in linkage group III has not been tested, locus designations are not used for the other CI-8AG strains. Studies to map the *aza-3* gene within linkage group III, as well as to evaluate allelism in a series of azaguanine resistant mutants, are now in progress.

To determine whether the new genes are dominant or recessive, vegetative heterokaryons were formed. Conidia from a CI-8AG strain and a strain with the A, *ad-3B*, *al-2*, *cot*, and *pan-2* markers were transferred to adenine-supplemented Fries conidiating medium lacking the supplementation for either homokaryotic strain but permitting the two strains to complement and grow as a heterokaryon. Conidia from heterokaryons of the CI-8AG strains did not form colonies in Westergaard's plating medium containing limited adenine (2.0 $\mu\text{g/ml}$) and azaguanine (200 $\mu\text{g/ml}$). Although at high conidial concentrations background growth in the presence of azaguanine is slightly heavier in the CI-8AG-carrying heterokaryons than in fully sensitive heterokaryons, it was concluded that azaguanine resistance is recessive to sensitivity.

DISCUSSION

Reports from the literature vary considerably concerning the effectiveness of azaguanine as an inhibitor and the reversibility of its effects by adenine. While azaguanine concentrations of the order of 1 to 30 $\mu\text{g/ml}$ are effective in mammalian tissue cultures (Chu, 1971; Chu, Brimer, Jacobson & Merriam, 1969), higher concentrations are required for inhibition in fungi. A concentration of 200 $\mu\text{g/ml}$ was found to be effective in *Aspergillus* (Morpurgo, 1962) and in *Schizosaccharomyces* (Heslot *et al.* 1966). Bedair & Fuerst (1961) reported that 350 μg azaguanine/ml completely inhibited growth of *Neurospora*. The relative ineffectiveness of azaguanine described here is closer to that found by Jha (1971).

The antagonism of azaguanine-induced inhibition by adenine sulphate is consistent with the findings of Morpurgo (1962) and Jha (1971). Szybalski & Smith (1959), on the other hand, found that adenine did not interfere with the effect of azaguanine in mammalian cells. Since adenine or hypoxanthine can serve as the sole purine source in adenine auxotrophic strains of *Neurospora* (Fairley & Loring, 1949), it would be reasonable to expect that adenine reverses the inhibitory action of azaguanine by serving as a source of normal guanylic acid. The effect of adenine sulphate on inhibition could also be explained by competition for transport into the hyphae. That adenine can antagonize the uptake of guanine has been demonstrated in *Schizosaccharomyces* by Pourquie (1970).

In each CI-8AG strain, the resistance marker was recessive to its sensitive allele. Incorporation of an *aza* gene into a heterokaryon, however, conferred upon the heterokaryon a slight decrease in sensitivity to azaguanine. Possible explanations are that the *aza* genes may be less than fully recessive, or that the inhibitory medium selects for a heterokaryon nuclear balance favouring the resistant component. Recessiveness of the azaguanine-resistance genes is consistent with reports for azaguanine resistance at other loci and in other organisms. The *aza-1* and *aza-2* genes in *Neurospora* are recessive in heterokaryons (Jha, 1971). Reces-

siveness has also been demonstrated for azaguanine resistance in Chinese hamster cells in tissue culture (Chu *et al.* 1969) and in *Aspergillus* (Morpurgo, 1962).

The potential use of azaguanine sensitivity and resistance as a system for the study of chemical mutagenesis was suggested by Szybalski (1958). Morpurgo (1962) reported the use of azaguanine resistance for determining forward mutation frequencies in *Aspergillus*. Chu & Malling (1968) developed a mutation system in which both forward mutation to azaguanine resistance and reversion to sensitivity can be detected in Chinese hamster cell cultures. The *aza-3* gene in strain CI-8AG no. 6 is being used in the development of a new mutation detection system in a *Neurospora* heterokaryon (Hoffmann & Malling, 1974).

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