Abnormalities in Cell Division Induced by Diepoxybutane in *rad1-1* and *rad3* Mutants of *Saccharomyces cerevisiae*

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Saccharomyces cerevisiae mutants *rad1-1* and *rad3* differ from the wild-type and from other UV-sensitive *rad* mutants in their behaviour after transfer from medium containing 1,2:3,4-diepoxybutane (DEB) to DEB-free medium. In both mutants several post-treatment cell cycles proceed in the absence of cell wall separation, resulting in the formation of multicellular chains or aggregates. In this study, electron and light microscopy revealed that at least one post-treatment budding cycle is accompanied by nuclear division while subsequent cell cycles can proceed in the absence of regular nuclear cycles. At low percentage survival levels, the first post-treatment budding cycle was not delayed and was accompanied by significant incorporation of radioactivity into DNA and protein. In contrast, subsequent cell cycles were found to be accompanied by only protein synthesis and not DNA synthesis. The wild-type strain, unlike the mutants, responded to DEB treatment by a dose-dependent lag in the onset of macromolecular synthesis and cell proliferation, and after prolonged incubation in mutagen-free medium the culture consisted of single budded and unbudded cells.

INTRODUCTION

Saccharomyces cerevisiae rad mutants are extensively used in the study of repair of DNA damage by physical and chemical treatments. Our experiments on liquid holding recovery (LHR) have revealed that in the *rad3* mutant, DEB exposure induces characteristic abnormalities in the cell division cycle, which result in the formation of multicellular chains or aggregates (Swietlińska *et al.*, 1978). Similar abnormalities have been observed in *rad1-1* and *rad7* mutants. In the present study we have undertaken to correlate cytological events with macromolecular synthesis and the LHR phenomenon.

METHODS

Strains and media. Haploid strains 197/2d (wild-type) and the *rad3* mutant (both $\alpha ade2-1$) were provided by B. S. Cox. A haploid *rad1-1* mutant was obtained from the Yeast Genetic Stock Center, Berkeley, USA. Complete medium (YPG) consisted of 1% (w/v) yeast extract, 1% (w/v) Difco Bacto-Peptone and 2% (w/v) glucose. Minimal medium was Yeast Nitrogen Base medium (Difco) without amino acids, containing 2% (w/v) glucose, supplemented with adenine at 20 $\mu\text{g ml}^{-1}$.

Mutagen treatment and post-incubation. Cultures were grown for 3 d in a complete liquid medium, harvested by centrifugation, washed twice with phosphate buffer (0.1 M-KH₂PO₄, pH 7) and re-suspended in the same buffer at 1×10^8 cells ml^{-1} . Treatment with 1,2:3,4-diepoxybutane (DEB; Merck) was performed at 30 °C and terminated by centrifugation followed by two washes with buffer. In some experiments the treated cells were liquid-held for 4 d in the same buffer with vigorous shaking. For observation of post-treatment growth, cells were resuspended in liquid growth medium at a density of $3-6 \times 10^6$ cells ml^{-1} and kept at 30 °C with shaking.

Observations on cell division cycle. Cell proliferation was monitored by counting cell numbers in a haemocytometer. Cell morphology was examined in the electron microscope, or under the light microscope after staining with Giemsa according to Robinow (1975). For electron microscopy cells were fixed in a 1.5% (w/v) solution of

potassium permanganate according to Smith *et al.* (1972), or in osmium tetroxide and potassium permanganate. In the latter method pelleted cells were washed several times with distilled water and resuspended in 2% (w/v) aqueous solution of osmium tetroxide for 1 h at room temperature. Post-fixation was performed in a 2% (w/v) aqueous solution of potassium permanganate for 2 h at room temperature and followed by several washings with distilled water. All the following steps were carried out according to Smith *et al.* (1972). Material was embedded in Epon 812 (Luft, 1961) and cut with a glass knife on a Reichert ultramicrotome. The silver-coloured sections were stained with lead citrate (Reynolds, 1963) for 25 min and examined with a JEM 100C electron microscope at 80 kV.

Assay of DNA and protein synthesis. Macromolecular synthesis was estimated according to Slater (1973) with slight modifications in sample volume and labelling. DNA was labelled in minimal medium by addition of [6-³H]uracil giving 3 µCi ml⁻¹ (specific activity 16.3 Ci mmol⁻¹; 603 GBq mmol⁻¹) or [¹⁴C]uracil giving 0.3 µCi ml⁻¹ (specific activity 43.8 mCi mmol⁻¹; 1.621 GBq mmol⁻¹). Protein was labelled in minimal medium with [¹⁴C]leucine (specific activity 210 mCi mmol⁻¹; 7.77 GBq mmol⁻¹) giving 0.04 µCi ml⁻¹. Radioactivity was counted in a scintillation mixture of toluene/PPO/POPOP in a Packard Tricarb Spectrometer.

RESULTS

Cell division cycle in mutant strains

When stationary phase cells of the *rad1-1* and *rad3* mutants are exposed to DEB (survival level less than 15%) and then post-incubated in a liquid growth medium, budding is resumed by the whole population of treated cells and proceeds at the same initial rate as in the control cultures. Buds produced by the treated cells enlarge but do not separate from their mother cells, so that after 4–5 h there is an almost uniform population of double cells. Observation in the electron microscope revealed that during the first post-treatment budding cycle the nucleus migrated to the neck between the mother and the daughter cell (Fig. 1*a*). We concluded, however, that nuclear division was not blocked at this stage, because cells which had completed nuclear division were also observed (Fig. 1*b*).

In most cases both the mother and the daughter cell (still unseparated) initiated new buds, which as in the first post-treatment cell cycle remained attached to their parent cells. It can be seen in electron micrographs that cross-walls are formed between mother and daughter cells, but cell separation is blocked by defective cell wall separation (Fig. 2*a*). Budding can be continued for several cell generations, resulting in formation of multicellular chains or aggregates irregular in shape. Light microscope pictures of aggregates are shown in Fig. 2(*b, c*). In some cultures aggregates containing as many as 20 cells were observed. Electron micrographs revealed that aggregate formation was not only due to the lack of cell wall separation, but also to incomplete cytokinesis in some cell cycles (Fig. 2*d*). Cells which had not completed cytokinesis were, however, less common than those blocked in cell wall separation.

Analysis of electron micrographs suggested that during chain growth, some cell cycles were not accompanied by regular nuclear cycles. As a result, within an individual chain or aggregate, anucleate cells as well as nucleated ones could be formed. Cells lacking nuclei generally occupied a terminal position in the chain or aggregate, but were also observed between nucleated cells. The latter observation suggests that in some cell cycles the nucleus is able to migrate from the mother to the daughter cells in the absence of regular division. In such cases the mother cell would become anucleate, while the nucleate daughter cell is likely to be capable of further proliferation.

The possibility of nuclear migration was supported by analysis of nuclear cycles in the light microscope after Giemsa staining. Although we were unable to obtain well-contrasted pictures of aggregate cells (whose cytoplasm was more dense than that of the control exponential phase cells) by this method, it was possible to determine the position of the nuclei in whole cells. We observed, within individual multicellular aggregates, both nucleated and anucleate cells as well as nuclei located in the isthmus between two neighbouring cells.

Rate of cell proliferation and colony-forming ability

The rate of cell proliferation was analysed by scoring the cell number in a haemocytometer during post-treatment incubation in growth medium. The frequencies of single cells and aggre-

Fig. 1. First post-treatment cell division cycle in a DEB-exposed *rad3* mutant. (a) The arrow indicates localization of the nucleus in the neck between the mother and the daughter cell. (b) The arrows indicate nuclear division. The bar markers represent 1 μm .

gates differing in cell number, scored after 5, 10 and 20 h of incubation, are given in Table 1. It is clear that control cultures of both wild-type and mutant strains proliferated as single cells. The strains differed only in generation time, with that of *rad3* longer than that of the wild-type, with *rad1-1* showing the shortest generation time.

Fig. 2. Abnormalities in cell division induced by DEB treatment. (a) Aggregate of *rad1-1* mutant. The arrows indicate the lack of cell wall separation. The bar marker represents 2 μm . (b) and (c) light microscope pictures of aggregates of *rad3* mutant after staining with potassium permanganate. The bar marker represents 7 μm . (d) Aggregate of *rad1-1* mutant. The arrows indicate cells with incomplete cytokinesis. The bar marker represents 2 μm .

Wild-type cells exposed to a high DEB dose (0.4%, v/v) respond to this treatment by a decreased rate of cell proliferation in mutagen-free medium. After 20 h the number of cells only doubles and single unbudded cells are most frequent while double cells or aggregates are rare. As shown in Table 1, mutant strains were exposed to much lower DEB doses which did not cause

Table 1. Cell proliferation in the wild-type (197/2d) and mutant strains during post-treatment incubation in DEB-free medium (YPG)

The initial cell number is indicated at zero time. Survival: 197/2d 20.1%; *rad1-1*, plated immediately 2.7%, after liquid holding 34.5%; *rad3*, plated immediately 10.0%, after liquid holding 58.1%.

Strain and treatment	Time in YPG (h)		10 ⁻⁵ × No. of single cells ml ⁻¹		10 ⁻⁵ × No. of aggregates ml ⁻¹			
	Plated immediately	After liquid holding	Unbudded	Budded	2-cell	3-cell	4-cell	Multicellular
197/2d (control)	0		25	9				
	6		72	59				
	0		29	6				
	6		25	9				
	12		13	11	7	1		
197/2d (DEB 0.4%, 45 min)	20		44	11	1	1		
	0		40	2				
	5		18	128				
	0		48	2				
	5			4	47			
<i>rad1-1</i> (control)	10		5		23	6	10	4
	20			60	15	15	32	10
	0		40	1				
	5		8	5	26			
	10		4	7	12	1	10	3
<i>rad1-1</i> (DEB 0.05%, 35 min)	20		200	210	39	10	52	43
	0		30	2				
	5		53	12				
	0		46	3				
	5			9	33			
<i>rad3</i> (control)	10		1		31	2	5	1
	20			9	10	10	12	20
	0		50	3				
	5			14	47			
	10		1	10	17	8	6	8
<i>rad3</i> (DEB 0.05%, 30 min)	20		100	39	40	42	20	33
	0		50	3				
	5			14	47			
	10		1	10	17	8	6	8
	20		100	39	40	42	20	33

any significant delay in the first post-treatment budding cycle. After 5 h the population of *rad1-1* and *rad3* consisted mainly of double cells which, during prolonged incubation, gave rise to multicellular aggregates at a high frequency.

The *rad1-1* and *rad3* mutants show a high level of liquid holding recovery (LHR) after DEB exposure (Žuk *et al.*, 1978). To analyse the effect of liquid holding (LH) on cell proliferation, the treated cells were held in buffer for 4 d and then incubated in growth medium under the same conditions as for the sample not exposed to LH. When the treated cells were grown following the LH period, their rate of proliferation increased significantly, and after 20 h there were greater numbers of both multicellular aggregates and single cells as compared with the sample not exposed to LH (Table 1). It remains unclear whether the increase in the frequency of single cells is due to regular division cycles of some parental cells, or to separation of individual cells from the multicellular aggregates.

Colony-forming ability and its dependence on LH was studied in the *rad3* mutant by means of isolation of single cells or multicellular aggregates, and plating them separately on agar. Although a rather low number of cells was isolated it was clear that without LH only few aggregates gave rise to colonies (Table 2). The same procedure revealed that following LH both single cells and aggregates formed colonies with increased frequency. The question arises as to how many cells in the aggregate contribute to the formation of a single colony. This question cannot be answered categorically but one may presume that one viable cell is sufficient to start colony formation.

Table 2. *Effect of liquid holding on colony forming ability in rad3 cells after exposure to 0.05% (v/v) DEB for 30 min*

Cells were incubated for 12 h in liquid growth medium directly after treatment, or after 4 d liquid holding. Colony forming units (c.f.u.) were then plated separately on nutrient agar.

Classes of colony-forming unit	Without liquid holding		After 4 d liquid holding	
	No. of c.f.u. isolated	No. of colonies	No. of c.f.u. isolated	No. of colonies
Single unbudded cells	0	0	4	3
Single budded cells	5	1	4	4
3-cell	4	0	3	2
4-cell	2	1	3	2
Multicellular	7	1	6	4

Fig. 3

Fig. 4

Fig. 3. Incorporation of radioactivity into DNA and protein of wild-type cells (197/2d). Filled symbols represent incorporation of $[^3\text{H}]\text{uracil}$ into DNA; open symbols represent incorporation of $[^{14}\text{C}]\text{leucine}$ into protein. $\circ, \bullet$, Control cells; $\square, \blacksquare$, cells exposed to 0.4% (v/v) DEB for 30 min (cell survival 38.2%); $\triangle, \blacktriangle$, cells exposed to 0.4% (v/v) DEB for 45 min (cell survival 20.7%).

Fig. 4. Incorporation of radioactivity into DNA and protein of *rad1-1* cells. Filled symbols represent incorporation of $[^{14}\text{C}]\text{uracil}$ into DNA; open symbols represent incorporation of $[^{14}\text{C}]\text{leucine}$ into protein. $\circ, \bullet$, Control cells; $\square, \blacksquare$, cells exposed to 0.05% (v/v) DEB for 30 min (cell survival 7.7%).

Macromolecular synthesis

In the wild-type cells a high DEB dose (0.4% v/v), inhibits incorporation of radioactivity into both DNA and protein during post-treatment incubation in mutagen-free medium (Fig. 3). This inhibition is dose-dependent. At a survival level of 38.2%, incorporation into DNA is delayed for 6 h, while at a lower survival level (20.7%), it is not resumed during a 12 h incubation period. A similar but less pronounced delay was observed for incorporation of radioactivity into protein (Fig. 3).

The *rad1-1* and *rad3* mutants are highly sensitive to DEB, hence they were exposed to much lower doses (0.05%, v/v). We found that in mutant cells, unlike the wild-type, incorporation of radioactivity into DNA began shortly after replacement in mutagen-free medium. As shown in Fig. 4, with *rad1-1* at a survival level of 7.7%, the incorporation of radioactivity into DNA was quite significant after 2 h of post-treatment incubation. It fell, however, at 4 h, and did not increase during the 12 h incubation period. In contrast, incorporation of radioactivity into protein remained at a high level throughout the 12 h incubation.

Incorporation of radioactivity into the DNA of DEB-treated *rad3* cells shortly after replacing them in mutagen-free medium has been reported elsewhere (Swietlińska *et al.*, 1978; Žuk *et al.*, 1980). Thus in both mutants, DEB does not seem to inhibit the first post-treatment round of DNA synthesis. Strong suppression of DNA replication is observed, however, during further incubation in growth medium and this phenomenon is obviously responsible for the high sensitivity of the mutants towards DEB.

DISCUSSION

We have found that in the *rad1-1* and *rad3* mutants, DEB treatment does not result in inhibition of the first post-treatment round of DNA synthesis, and induces abnormalities in the cell division cycle leading to the formation of multicellular aggregates. Such post-treatment behaviour differed from that of the wild-type strain, which responded to DEB with a dose-dependent suppression of DNA synthesis and cell proliferation.

Polyfunctional alkylating compounds (DEB is actually bifunctional) are known to introduce, into yeast DNA, inter- and intra-strand cross-links, and several monofunctional alkylation products, as well as single-strand breaks, in a dose-dependent manner (Kircher *et al.*, 1979; Fleer & Brendel, 1979; Žuk *et al.*, 1980). Mutants *rad1-1* and *rad3* are highly DEB-sensitive: a tenfold lower dose is sufficient to reduce cell survival to levels comparable with those of wild-type cells (Žuk *et al.*, 1978). Therefore, one can assume that the incorporation of radioactivity into DNA and protein in both mutants shortly after returning the cells to mutagen-free medium is a feature of the very low doses of DEB and the few lesions induced. On the other hand, wild-type cells were exposed to much higher doses, which suppressed both DNA and protein synthesis.

Undelayed incorporation of radioactivity into DNA after DEB treatment was also noted for *rad2*, *rad4* and *rad7* mutants (unpublished data). These strains, like *rad1-1* and *rad3* are excision-deficient (Prakash, 1975, 1977; Reynolds, 1978) and show an increased sensitivity towards DEB (Žuk *et al.*, 1978). On the other hand, we have found that in another highly DEB-sensitive strain, *rad18-2* (Žuk *et al.*, 1978) incorporation of radioactivity into DNA was strongly suppressed by low DEB doses while protein synthesis was almost unaffected. It should also be noted that in this strain we did not observe aggregate formation (unpublished data). Since *rad18-2* is blocked in a pathway other than excision repair (Lawrence & Christensen, 1976) we suggest that the undelayed incorporation of radioactivity into the DNA of DEB-exposed *rad1-1* and *rad3* cells is not only due to low doses of the drug applied, but also to deficiency in excision repair.

The question arises, whether aggregate formation is also characteristic of other excision-deficient *rad* mutants. We analysed cell proliferation after DEB exposure in all UV-sensitive *rad* mutants of the series *rad1* to *rad21* (except *rad13*). Only *rad7* (also excision-deficient) formed multicellular aggregates similar to those described here. In two other excision-deficient strains, *rad2* and *rad4*, DEB induces double cell formation, but further proliferation is stopped after one additional cell cycle (unpublished data). Thus deficiency in excision repair cannot be the only cause of the abnormal cell division cycle observed in *rad1-1* and *rad3* strains after DEB exposure.

It is known that in yeast cells, inhibition of DNA synthesis by several treatments such as hydroxyurea (Slater, 1973), Trenimon (Jaenicke *et al.*, 1970) or X-rays (Eckstein *et al.*, 1967) does not result in simultaneous inhibition of budding. Slater (1973) reported, however, that inhibition of protein synthesis by anisomycin prevented budding of cells exposed to hydroxy-

urea. This finding is consistent with our observation that budding was suppressed only in wild-type cells in which both DNA and protein synthesis were inhibited by high DEB doses.

Continuation of budding with DNA synthesis suppressed is also characteristic of conditional cell division cycle mutants (*cdc*) grown at the restrictive temperature (Hartwell, 1971). The mutant *cdc4*, defective in initiation of DNA synthesis, develops as many as five buds on a single mononucleate cell. Hence, in this respect it does not resemble the *rad* mutants described here. The formation of multicellular aggregates by *rad1-1* and *rad3* after DEB exposure is also different from the mode of proliferation of *cdc* mutants with defective cytokinesis. The latter, at the restrictive temperature, undergo multiple rounds of DNA replication in the absence of cytokinesis or cell wall separation (Hartwell *et al.*, 1974). According to the present data, only the first post-treatment budding cycle in *rad1-1* and *rad3* was accompanied by DNA synthesis; subsequent cell division cycles could proceed in the absence of regular nuclear cycles.

The present data reveal that the high increase in survival of DEB-treated *rad1-1* and *rad3* cells observed after liquid holding is not related to the aberrant mode of cell proliferation. Liquid holding was found to increase colony formation by both single cells and aggregates. This suggests that liquid holding enhances DNA repair and possibly ensures more regular nuclear cycles, but does not affect cell separation.

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