R Factor Elimination by Inhibitors of Thymidylate Synthetase (Fluorodeoxyuridine and Showdomycin) and the Occurrence of Single Strand Breaks in Plasmid DNA

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Summary. Fluorodeoxyuridine is bactericidal and actively eliminates R factor 1818 from Escherichia coli; both effects are prevented by the addition of thymidine. R factors 7268 and TEM are not eliminated by fluorodeoxyuridine treatment. Showdomycin is bactericidal but does not eliminate R-1818. Thymidine only partially protects showdomycin-treated cultures. Fluorouracil eliminates R-1818 at one third the rate of fluorodeoxyuridine from bacteria that are wild-type with respect to thymidine phosphorylase, but is much less effective than fluorodeoxyuridine in curing R-1818 from a thymidine phosphorylase-deficient mutant. The amount of R-1818 DNA present in the closed circular form decreases from 1% of chromosomal DNA in control cultures to 0.35% after three hours treatment with fluorodeoxyuridine; under similar conditions the amount of R-7268 DNA remains virtually constant. Showdomycin treatment apparently increases the proportion of closed circular R-1818 DNA. The results are discussed in terms of pyrimidine metabolism and it is suggested that R-1818 elimination by fluorodeoxyuridine treatment results from the induction, by thymineless conditions, of an R factor-mediated endonuclease.

Introduction

Following an observation that thymine starvation eliminates certain R factors from thymineless mutants of Escherichia coli, Klebsiella aerogenes and Salmonella typhimurium (Pinney and Smith, 1971 and unpublished results), we have demonstrated that trimethoprim, which blocks the enzyme dihydrofolate reductase and therefore induces thymineless conditions in wild type strains (Cohen, 1971), is also a curing agent (Pinney and Smith, 1973). We now report the genetic and molecular effects on R factors produced by compounds that block thymidylate synthetase, the enzyme responsible for the final step in the synthesis of thymidylate acid (Cohen, Flaks, Barner, Loeb and Lichtenstein, 1958).

5-Fluorodeoxyuridine is converted in vivo to 5-fluorodeoxyuridine monophosphate by thymidine kinase (O'Donovan and Neuhard, 1970) and the latter nucleotide has been shown to irreversibly inhibit thymidylate synthetase (Cohen et al., 1958). Showdomycin, as well as inhibiting uridine monophosphate kinase and uridine phosphorylase (Roy-Burman, Roy-Burman and Visser, 1968), also irreversibly inactivates thymidylate synthetase in vitro (Kalman, 1972). These two compounds were therefore tested for their R factor curing ability.

We report that fluorodeoxyuridine eliminates R factor 1818 from exponential cultures of E. coli and that the proportion of R factor molecules in the covalently
closed circular form decreases by more than 60% during fluorodeoxyuridine treatment. Showdomycin, however, does not produce elimination of the R factor. A preliminary report of some of this work has been published (Bremer, Pinney and Smith, 1973).

Material and Methods

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*Bacterial Strains and R Factors.* Escherichia coli strain J5-3 pro-met and R factors 1818, TEM and 7268 have been described previously (Pinney and Smith, 1971; 1973). E. coli K12SH-28, a thymidine phosphorylase-deficient mutant (Fangman and Novick, 1966), kindly supplied by Dr. Walton L. Fangman, was also used.

**Media.** These have been described previously (Pinney and Smith, 1973).

**R Factor Elimination.** Exponential phase cultures were grown to approximately \( 2 \times 10^8 \) organisms per ml in suitably supplemented Davis Mingioli medium (DM) (Davis and Mingioli, 1950). The cells were spun down and resuspended in fully supplemented DM at 37\(^\circ\)C containing 100 \( \mu \)g fluorodeoxyuridine (Calbiochem) or 10 \( \mu \)g showdomycin (Calbiochem) per ml. Samples were taken at intervals, diluted in nutrient broth and plated on MacConkey agar. After overnight incubation, suitable clones were replica plated to test for R factor retention (Pinney and Smith, 1971).

**R Factor DNA Determination.** Overnight cultures, grown in DM, were subcultured 1 in 10 into fully supplemented DM containing 10 \( \mu \)Ci/ml [methyl-\( ^3 \)H] thymidine (specific activity 5.0 Ci per mmole, supplied by the Radiochemical Center, Amersham) and 290 \( \mu \)g deoxyadenosine per ml. The subcultures were shaken at 37\(^\circ\)C for 150 min then centrifuged (4000 r.p.m., 15 min). The labelled cell pellets were either washed immediately in 0.01 M phosphate buffer pH 7.0 containing 0.001 M MgSO\(_4\), 0.0001 M CaCl\(_2\) and 0.1 M NaCl and lysed (control cultures), or resuspended in fully supplemented DM containing fluorodeoxyuridine or showdomycin for various times before washing and lysis. Cultures were lysed and prepared for alkaline sucrose gradient centrifugation essentially by the method of Freifelder, Folkmanis and Kirschner (1971). Washed cells were resuspended at 37\(^\circ\)C in 0.2 ml of a lysis buffer, which consisted of 0.05 M NaCl and 0.02 M ethylenediaminetetraacetic acid (EDTA) in 0.02 M tri(hydroxymethyl)aminomethane buffer pH 9.1, in a 0.5 drachm shell (Johnson and Jorgensen Ltd. London, S.E.7.). To lyse the cells, 0.2 ml of a 1% solution of sodium dodecyl sulphate in 0.8 M NaOH at 37\(^\circ\)C was added over a period of 40 sec with gentle stirring. The lysate was vortexed for 30 sec to fragment chromosomal DNA, then layered onto a 12.5 ml 5-20% linear alkaline sucrose gradient containing 0.3 M NaOH, 1.0 M NaCl and 0.02 M EDTA. Gradients were centrifuged at 38000 r.p.m. for 65 min in the S40/135 rotor of a Griffin-Christ Omega II ultra-centrifuge at 20\(^\circ\)C. 0.25 ml fractions were collected by upward displacement using an Isco model 183 fractionator linked to an Isco model 563 fraction collector. Samples were precipitated with 5% cold trichloroacetic acid (TCA) and collected on glass fibre discs (Whatman GF/C). They were washed with 1 ml quantities of cold 5% TCA and water, and dried in vacuo over silica gel. 5 ml of scintillant consisting of 6 g 2,5-diphenyloxazole, 75 mg 1,4-bis-[2-(5-phenyloxazolyl)]-benzene, 1 l toluene and 500 ml ethyl alcohol was added and radioactivity determined in a Packard model 574 liquid scintillation spectrometer.

**Results**

**Effect of Fluorodeoxyuridine and Showdomycin on Bacteria Harbouring R 1818.** The decline in viability of strain J5-3 in 100 \( \mu \)g of fluorodeoxyuridine per ml was similar to that in 10 \( \mu \)g of showdomycin per ml and comparable with that produced by thymine starvation of a thymineless mutant of strain J5-3. Possession of R 1818 made little difference to the survival of cells in showdomycin (Fig. 1) but gave a marked protective effect against fluorodeoxyuridine (Fig. 2). A small number of showdomycin-resistant cells were present in all cultures tested, and these grew in the concentration of showdomycin used. After overnight incubation,