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Isolation and characterization of bleomycin-resistant clones of CHO cells

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SUMMARY

Five clones of Chinese hamster ovary cells with increased resistance to bleomycin have been isolated following ethylmethanesulphonate mutagenesis. Resistance was stable in three of the clones, but unstable in the other two. One of the stably resistant clones was cross resistant to unrelated drugs, and in contrast to the parental cells, its response to bleomycin was potentiated by tween 80. These two observations suggested a membrane alteration in the resistant clone. There was no significant difference in bleomycin-inactivating enzyme activity between the parental and resistant clones.

1. INTRODUCTION

Bleomycin is a glycopeptide antibiotic complex which has significant antitumour activity, notably against squamous cell carcinoma cells *in vivo* (Crooke & Brather, 1976; Blum, Carter & Agre, 1973). Bleomycin binds to DNA (Muller & Zahn, 1977) and induces strand scission and release of thymine residues (Muller *et al.* 1972). The presence of bleomycin reduces the activity of DNA and RNA polymerases *in vitro*, but *in vivo* only a significant effect on DNA synthesis is seen (Muller *et al.* 1975).

Cell cycle studies indicate that bleomycin induced blockage is often during the G2 phase of the cell cycle at concentrations of bleomycin which are insufficient to produce inhibition of DNA synthesis (Nagatsu, Richart & Lambert, 1972). In CHO cells, for example, the cell cycle has been shown to be blocked one hour before mitosis, at a point lying between the temporal markers representing the final RNA species and the final protein species required for division (Tobey, 1972). In mouse L cells, however, it is also observed that the S phase is lengthened, prior to blockage in G2 (Wanatabe *et al.* 1974).

Bleomycin induces a number of kinds of chromosome damage in several cell lines *in vitro*, including CHO cells (Hittelman & Rao, 1974). There is evidence that chromosome damage can occur during G1, S and G2, but the major part of the damage appears to be induced during S phase (Paika & Krishnan, 1973).

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The chromosomal damage appears to be responsible for subsequent delay in the G2 phase.

Studies of bleomycin resistance may provide insight into the site of action of the drug or into patterns of cross resistance which may be relevant in design of effective chemotherapy schedules. Saunders & Schultz (1972) have isolated bleomycin-resistant strains of *Bacillus subtilis* and Satoh and Shinura (1969) have observed *in vivo* resistance of a rat ascites hepatoma. In this paper we describe the isolation and partial characterization of stable bleomycin-resistant clones of Chinese hamster ovary cells following exposure to the mutagen ethylmethanesulphonate *in vitro*.

2. MATERIALS AND METHODS

(i) *Cell lines, media, chemicals*

A hypodiploid line of Chinese hamster ovary cells, designated CHO-K1 (Kao & Puck, 1968) was used. Although the Chinese hamster has 22 chromosomes, this cell line has a modal chromosome number of 20. The cell line was kindly provided by Dr R. Wilson of the Genetics Department, University of Glasgow. Cells were grown in Glasgow modification Eagles medium with 10% foetal calf serum (both from Flow laboratories). Hanks balanced salt solution used in this work was from Gibco-Biocult Ltd. and sterile freeze dried trypsin was from Wellcome Reagents Ltd. For drug response studies, plates were incubated for seven days and then stained with Leishman stain. Colonies with over 50 cells were counted as viable.

Other chemicals were from the following sources: Bleomycin, clinical grade (a mixture of bleomycin A2 and B2) from Lundbeck Ltd; ethylmethanesulphonate and actinomycin D from Sigma; vinblastine sulphate ('Velbe') from Eli Lilly; colchicine from British Drug Houses; the diagnostic sensitivity test agar (DST agar) from Oxoid Ltd.

Karyotype analysis was carried out on cells treated with 10 μg/ml colchicine for 4 hours. Following treatment with 1·12% sodium citrate for 5 min. the cells fixed in ice cold methanol/acetic acid (3:1) and stained with aceto-orcein.

(ii) *EMS mutagenesis*

For each mutagenesis run, around 16 60 mm petri dishes, each containing around 5×10^4 cells, received 300 μg/ml ethylmethanesulphonate for 16 h. This EMS treatment resulted in around 11% survival. Following a period of 48 h in fresh drug-free medium, cells were exposed to 10 μg/ml bleomycin for 7 days. After this time cells were examined for surviving colonies and these were cloned using cloning rings and kept for further study.

(iii) *Potentiation of effects of bleomycin by tween 80*

The technique developed by Riehm and Biedler (1972) for studying the potentiation of actinomycin D by tween 80 in CHO cells was used. The aim of the experiment was to compare inhibitory concentrations of the drugs when in combination with inhibitory concentrations of the drugs alone. Dose response curves for cells

in bleomycin alone, in tween 80 alone and in bleomycin in the presence of several concentrations of tween 80 were determined. Comparisons between different platings were made in terms of ED_{80}s ('effective dose 80' the dose which gives 80 % cell death). Data for the drugs in combinations is expressed in terms of fractional inhibitory concentrations (FICs). The FIC for bleomycin in the presence of tween was obtained by dividing the ED_{80} for bleomycin in the presence of a certain concentration of tween by the ED_{80} in the presence of bleomycin alone. Conversely the FIC for tween in the presence of bleomycin was obtained by dividing the ED_{80} for tween in combination with bleomycin by the ED_{80} for tween alone. For example, a cell line may have an ED_{80} for bleomycin alone of 1·725 mg/ml and an ED_{80} for tween alone of 0·018 % (v/v). In the presence of 0·005 % tween the ED_{80} for bleomycin may be observed to be 1·330 mg/ml. The FIC for bleomycin is therefore 0·771 with a corresponding FIC for tween of 0·278. Data of this kind is plotted using the FIC of tween as the abscissa and the FIC of bleomycin as the ordinate. A straight line plot connecting 1·0 on the ordinate with 1·0 on the abscissa indicates that the two drug effects are simply additive. Deviation to the left of this line indicates synergism (potentiation) and deviation to the right indicates antagonism between the two substances.

(iv) *Measurement of bleomycin-inactivating enzyme activity*

Approximately 10^8 cells, grown in suspension culture, were harvested by low speed centrifugation, washed with 20 ml of 70 mM pH 7·0 phosphate buffer and then resuspended in 3·0 ml of the same buffer. The cells were then disrupted by sonication. In early experiments the sonicate was centrifuged at 105000 *g* for 60 min. prior to use, but when this step was found not to affect the results obtained, it was eliminated and enzyme assays were carried out directly on the sonicate. The assay of bleomycin-inactivating enzyme activity was carried out according to the method of Miyaki *et al.* (1975). 0·02–0·20 ml of sonicate was incubated at 37 °C for 60 minutes with 50 μg bleomycin in 30 mM phosphate buffer, pH 7·0, containing 60 mM sodium chloride. At the end of this time, the residual bleomycin was assayed by measurement of its growth inhibitory capacity using Klebsiella 'Southmede' as a test organism. 1·0 ml of a stionary phase culture, mixed with 20 ml of distilled water, was flooded over 100 ml of Oxoid DST agar in a 10 in square assay plate. The plate was drained and allowed to dry. Wells were punched in the agar with a number 5 cork borer and filled with a random array of test samples and standards in triplicate. The plates were incubated without lids for 30 min at 34 °C and then further incubated with lids overnight. The diameters of the inhibitory zones produced by test samples and standards were then measured and compared. Protein estimation was by the method of Lowry *et al.* (1951) using bovine serum albumen as a standard.

3. RESULTS

(i) *Selection and preliminary characterization of bleomycin-resistant clones*

Preliminary experiments indicated that exposure to 10 μg/ml bleomycin for 7 d gave survival of about 0·09 % and this concentration was used for selection of resistant clones. Seven mutagenesis runs were performed as described in the Materials and Methods section. These runs yielded a total of five resistant clones, designated blm_A through to blm_E. All of these clones were from separate mutagenesis

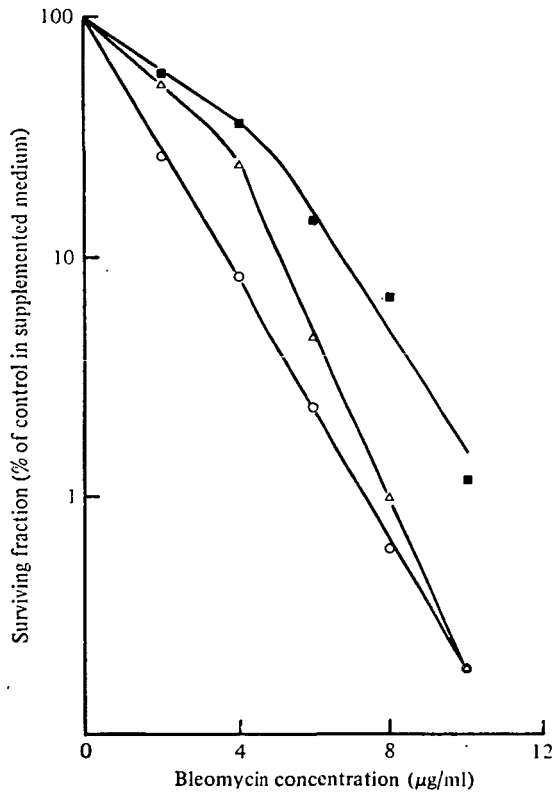

Fig. 1. Bleomycin resistance of blm_A and blm_D compared with parental cell cultures. ○—○, parental cell line; △—△, blm_A; ■—■, blm_D.

runs, except for blm_B and blm_C, which were derived from the same experiment. blm_C had very long, spindly cells, where blm_B had normal morphology. It was therefore considered quite probable that they were independent isolates although they came from the same mutagenesis run, and both were kept for further study. No stably bleomycin-resistant clones were isolated without mutagenesis.

All five of the clones were only around twice as resistant as wild type (Figs.1 and 2). This is a relatively slight increase in resistance, but it was consistently reproducible. In each case a marked shoulder was observed in the survival curve which was not found in wild type (i.e. the parental cell line) and, as far as we are

aware, has not been observed in survival curves of other cell lines exposed to bleomycin.

The average plating efficiency in drug free medium of blm_A, blm_B and blm_C was 64, 66 and 59 % respectively, approaching that of the parental cells (78 %). blm_D and blm_E however, had plating efficiencies of only 20 % and 34 % respectively.

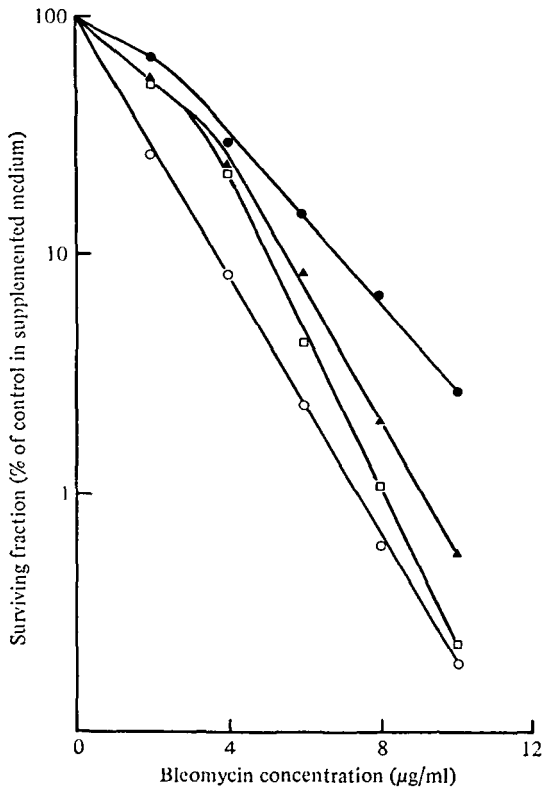

Fig. 2. Bleomycin resistance of blm_B, blm_C and blm_E compared with parental cell cultures. ○—○, parental cell line; ▲—▲, blm_B; □—□, blm_C; ●—●, blm_E.

The original CHO strain used for selection in this work had a modal chromosome number and 20 and the karyotype closely resembled that described by Kao and Puck (Fig. 3*b* in Kao & Puck, 1970). In contrast, three of the bleomycin-resistant clones (blm_A, blm_B and blm_C) had a modal chromosome number of 21. The two other resistant clones both initially showed a bimodal chromosome distribution, with some approximately diploid cells with a modal chromosome number of 20 and some cells with approximately 25–42 chromosomes. On subculturing blm_D over some months in drug-free medium, the pseudotetraploid cells completely outgrew the pseudodiploid strain, leaving only pseudotetraploid cells with a modal chromosome number of 38. blm_E maintained a bimodal distribution of around 70 % pseudodiploid and 30 % pseudotetraploid cells on subculturing.

At the end of 11 months subculturing in drug-free medium, the levels of resistance of blm_A, blm_B and blm_C were very similar to when they were first isolated. In contrast, blm_D had lost its resistance completely and blm_E was only slightly more resistant than wild type. Analysis of subclones of blm_E showed that both pseudodiploid and pseudotetraploid subclones both had similar low levels of resistance. In view of the instability of karyotype and resistance levels of blm_D and blm_E, only blm_A, blm_B and blm_C were further studied in detail.

(ii) *Evidence for a membrane change in blm_A*

Drug resistance in mammalian or other cells may occur by a variety of mechanisms, including altered uptake of the drug, altered breakdown of the drug or a change at the site of action of the drug. The following experiments were undertaken to try to provide clues to the mechanism(s) of resistance of blm_A, blm_B and blm_C.

Resistance conferred by membrane changes is often relatively non-specific and consequently cross resistance to unrelated drugs may be observed. The levels of cross resistance of the three clones to colchicine, vinblastine sulphate, actinomycin D and puromycin were therefore measured. blm_A was more resistant than the parental strain to vinblastine and puromycin, although the resistance of blm_B and of blm_C to these drugs was not distinguishable from the parental strain (Figs. 3, 4). Resistance of all three of the bleomycin resistant clones to colchicine and actinomycin D was indistinguishable from that of the parental strain.

A second type of experiment was carried out to investigate the possibility of membrane changes in these clones. These experiments study the interaction of tween 80 and bleomycin effects on parental bleomycin resistant strains. Tween 80 is a non-ionic surface active detergent which increases the permeability of the cell membrane, enhancing the uptake of dyes and proteins. Riehm & Biedler (1972) have shown that it potentiates the action of actinomycin D and daunomycin in drug-resistant Chinese hamster cells, indicating that the drug resistance in such cells is a function of membrane impermeability. The experimental technique and the method of interpretation of data used for these experiments was similar to that used by Riehm and Biedler and has been described more fully in the Materials and Methods section. Dose response curves were obtained for the parental strain and for blm_A, blm_B and blm_C in bleomycin alone (0–4 μg/ml) and with concentrations of tween up to 0·02% (v/v). Fractional inhibitory concentrations were calculated and the bleomycin fractional inhibitory concentrations were plotted against the tween fractional inhibitory concentrations. The parental cell line gave a straight line connecting unity on the abscissa and ordinate. This indicated that the effects of bleomycin and tween were purely additive and there appeared to be no membrane barrier to bleomycin entry that could be overcome by the use of tween in the parental cell line. A similar result was obtained with blm_B and blm_C. However, the plot for blm_A deviated markedly from a straight line (Fig. 5) indicating potentiation of bleomycin action by tween in these cells. At the point of maximum effectiveness of the two agents, given by the intersection of the

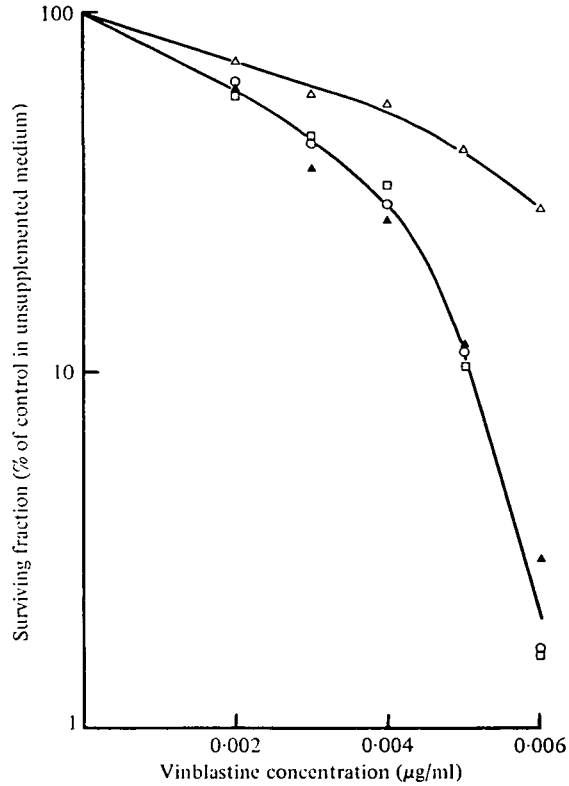

Fig. 3. Cross resistance of blm_A to vinblastine sulphate. ○—○, parental cell line; △—△, blm_A; ▲—▲, blm_B ;□—□, blm_C.

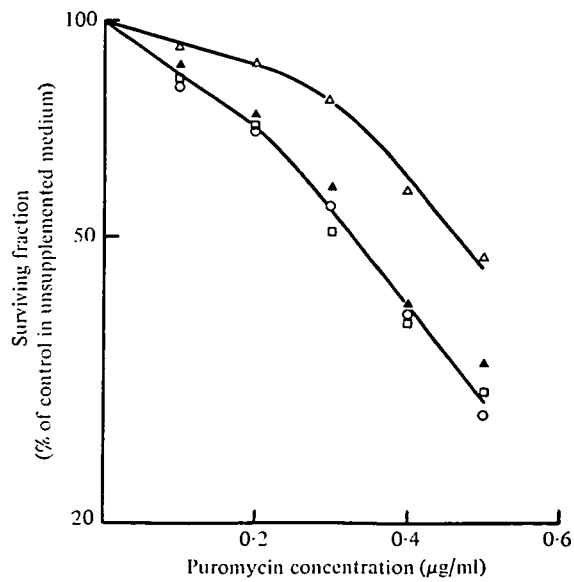

Fig. 4. Cross resistance of blm_A to puromycin. ○—○, parental line; △—△, blm_A; ▲—▲, blm_B; □—□, blm_C.

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two straight lines drawn through the experimental points, the sum of the fractional inhibitory concentrations was 0·66. This is considerably below the value of 1·0 which represents the situation when no drug interaction is seen. It therefore appears that the blm_A cell line has a membrane barrier to bleomycin action which can be reduced by the presence of tween.

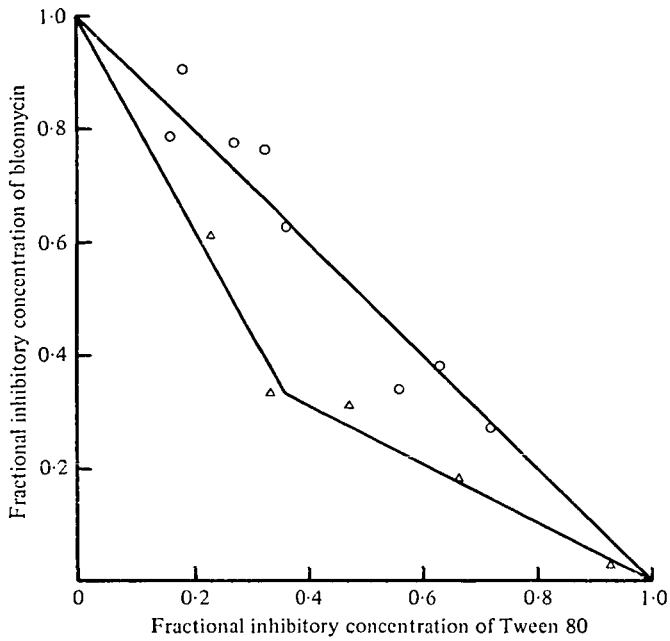

Fig. 5. Synergism of tween 80 and bleomycin on bleomycin resistant strain blm_A. ○—○, parental line; △—△, blm_A.

(iii) *Bleomycin-inactivating enzyme activity in parental and resistant cell lines*

It is conceivable that a cell line may become resistant to a drug by means of an increase in the activity of an enzyme which inactivates it. Bleomycin has been shown to be inactivated by an enzyme found in various tissues of mice (Umezawa, 1971). Studies on the products of inactivation indicate that the enzyme acts by cleavage of the carboxyamide bond of the β-aminoalanine part of the bleomycin molecule, liberating one mole of ammonia per mole of bleomycin. The enzyme is related to, but distinguishable from aminopeptidase B and it has been proposed that the enzyme may play a normal physiological role in the hydrolysis of basic proteins.

Bleomycin-inactivating enzyme activity was measured 3 or 4 times for the parental cell line and for blm_A, blm_B and blm_C (Table 1). It can be seen that there was no significant difference in the enzyme activity between any of the cell lines. The level of enzyme activity was similar to that of rat ascites hepatoma cells observed by Miyaki *et al.* (1975).

Table 1. *Levels of bleomycin inactivating enzyme activity in parental and resistant cell lines.*

Cell line	Number of experiments	Mean enzyme activity*	Standard Deviation
Parental	4	0·578	0·0174
blm_A	3	0·560	0·0731
blm_B	3	0·574	0·0226
blm_C	4	0·584	0·0340

* Activity is expressed in μg bleomycin inactivated/minute/mg protein

4. DISCUSSION

Five bleomycin resistant variants have been isolated in CHO cells. Many authors have pointed out that there is evidence for epigenetic as well as genetic mechanisms underlying the origin of variant clones in mammalian cell cutures. (See, for example, the discussion in Demars, 1974.) The resistance of three of our cell lines blm_A, blm_B and blm_C, was stable on subculture in drug free medium and such stability is generally taken as one line of evidence supporting a genetic origin for variant clones. Resistant clones were only isolated following EMS mutagenesis, which is also consistent with a genetic origin for our clones.

blm_D and blm_E showed neither stability of karyotype or of drug resistance. Terzi (1974) has described a class of bromodeoxyuridine-resistant BHK and Chinese hamster DON cells characterized by karyotype instability, low plating efficiency and high reversion frequency. He interpreted this class of mutants in terms of chromosomal variation generating variation in gene dosage and hence phenotypic instability. As blm_D and blm_E showed similar characteristics to these clones and tend to produce pseudotetraploid clones in which extensive re-arrangement could have occurred it might be speculated that gene dosage modulation of a basic phenotype was causing variation in apparent drug resistance in these lines. Although such unstable resistance is inconvenient for genetical study, it should be remembered that it may have clinical significance in the development of drug resistance during chemotherapy.

blm_A cells showed potentiation of drug action between tween 80 and cross resistance to puromycin and vinblastine sulphate, unlike blm_B and blm_C cells. By these criteria at least, part of the resistance of blm_A could therefore be attributed to an alteration in membrane permeability to the drug. Ling (1975) has described a general class of CHO mutants resistant to colchicine and to several other drugs. Possibly blm_A involves a similar defect in membrane organization to that in Ling's mutants, although we have not been able to detect any colchicine resistance in this clone.

blm_B and blm_C showed no cross resistance to any of the drugs tested and this does suggest that an alternative mechanism of resistance may be involved in these strains. One of the most obvious alternative hypotheses is that the increase in resistance could be due to an increase in bleomycin-inactivating enzymes, but

we were unable to detect any increase in activity in any of our clones. It may be noted that Miyaki *et al.* (1975) has shown that resistant rat ascites hepatoma cells had 3·5 times the enzyme levels of sensitive cells, the increase being sufficient to account for their decreased sensitivity to bleomycin. In contrast Ohnuma *et al.* (1974) observed no relationship between inactivation by neoplastic tissues and *in vivo* therapeutic effects in the case of two transplantable mouse tumours and several carcinomas and leukaemias in man.

An alternative hypothesis is that resistance in the case of blm_B and blm_C could involve alterations in the levels of DNA repair processes acting on DNA damage induced by the drug, especially since repair is considered to be a major factor in cell survival in the presence of bleomycin (Spangler *et al.* 1976). Studies of repair of single stranded breaks in sensitive and resistant cells would be of interest.

Although no positive evidence for the mechanism of resistance to blm_B and blm_C has been presented, the different behaviour of these cell lines to that of blm_A does show that the causes of bleomycin resistance in CHO-K1 cells cannot be attributed to one factor alone.

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