Comparison of the sequence specificity of bleomycin cleavage in two slightly different DNA sequences

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ABSTRACT

The sequence specificity of bleomycin damage was investigated utilising 340 bp α -DNA (a middle repetitive sequence in the human genome) as a target sequence. The following significant facts were found:- i) The dinucleotides GT and GC were cleaved on all occasions, GA most of the time, and AT, AC, GG and AA cleaved some of the time; ii) The base immediately 5' to the purine-pyrimidine dinucleotides was found to be statistically highly significant in determining the degree of damage caused by bleomycin, while other nearest neighbour bases had no significant effect; iii) The sequence specificity of bleomycin damage was determined on both strands and it was found that damage on either strand follows the above dinucleotide preference and is independent of the extent of damage on the opposite strand; iv) Bleomycin damage was compared between genomic 340 bp α -DNA and a cloned α -DNA with eleven base substitutions relative to the "consensus" sequence. There were forty-nine detectable differences in intensity of damage between these two DNA molecules. Although four of the differences can be directly attributed to changes in base sequence, the remaining differences were not at the base substitution sites. Some of the differences were over fifty base pairs from the nearest base substitution. We propose that the majority of these differences are due to microvariation in the structure of DNA with a slightly different DNA sequence.

INTRODUCTION

Bleomycin is a glycosidic antibiotic which is widely used in cancer chemotherapy (1,2). In intact cells and under appropriate conditions in vitro bleomycin causes damage to DNA. This DNA damage comprises single and double strand breaks and release of free bases and base-propenal from DNA. In vitro the reaction requires stoichiometric amounts of Fe^{2+} and the current evidence suggests that the reaction mechanism involves the oxidation of Fe^{2+} to Fe^{3+} with the concomitant production of reactive oxygen species which then damage the DNA (3-5).

Haseltine and co-workers (6-7) and Takeshita et al. (8) have studied the sequence specificity of bleomycin-induced single strand breaks in vitro using defined plasmid DNA sequences. They found that the dinucleotides 5' GpC 3' and GT are invariably cleaved, other sequences are cleaved occasion-

ally and some sequences are never cleaved. One intriguing aspect of their cleavage data is that for a particular dinucleotide the extent of damage can vary from one dinucleotide location to another. In this paper we attempted to further examine this question: firstly by evaluating bleomycin damage in both strands of a longer sequence of DNA and secondly, by comparing the cleavage patterns of slightly different DNA sequences.

The clinically used bleomycin preparation, Blenoxane (which was used in this study), is a mixture of several bleomycin species that differ in the terminal amine group - the main constituent bleomycin A₂ comprises approximately 70% of Blenoxane and bleomycin B₂ 30%. The sequence specificity of Blenoxane, bleomycin A₂ and bleomycin B₂ cleavage is the same (7).

The target sequence for our bleomycin experiments was 340 bp human α -DNA. This DNA species has been used to elucidate the sequence specificity of U.V. light (9) and nitrogen mustard (10). In humans 340 bp α -DNA comprises approximately 50,000 copies per haploid genome (about 1% of the total DNA) (11). Each DNA copy has a slightly different sequence (12,13) and this fact was exploited to produce a 340 bp α -DNA recombinant DNA clone in pBR325 (designated α -B3) with 11 base substitutions.

A system has been developed whereby α -DNA sequences are selectively purified from non- α -DNA (V. Murray and R. Martin - manuscript submitted for publication). This is accomplished using DNA-DNA hybridisation. A 340 bp sequence of α -DNA was cloned into bacteriophage M13. The single stranded M13 α -DNA clone is then hybridised to [³²P] 3'-labelled 340 bp α -DNA. Only one strand of the α -DNA hybridises to the M13 probe and the hybridised 340 bp α -DNA strand can then be purified by gel electrophoresis. Thus this method results in purified single strands (either (+) or (-) strands) which are labelled only at one end, so that after damaging the DNA with bleomycin, the sequence specificity and extent of damage can be assessed on both strands of the DNA. Since the method also results in the purification of α -DNA sequences from non- α sequences, damage in α -DNA can also be analysed in total cellular DNA after exposure to bleomycin.

MATERIALS AND METHODS

Materials All chemicals used were of reagent grade. All enzymes were purchased from either BRL, Boehringer-Mannheim or New England Biolabs. [α -³²P] dATP was from New England Nuclear. Bleomycin sulphate (Blenoxane) was from Bristol Laboratories.

Bleomycin reactions The treatment of DNA with bleomycin was carried out as

described by Kross *et al.* (7). Between 5 and 10 ng of 340 bp α -DNA was reacted with an appropriate concentration of bleomycin, an equimolar concentration of $\text{Fe}^{2+}\text{SO}_4$, and 25 mM tris, pH 7.5 for 30 minutes at 37°C. The samples were then immediately subjected to the M13 hybridisation procedure. When total human cell DNA was treated with bleomycin, approximately 10 μg of DNA was used. After the reactions were completed, the DNA samples were immediately loaded on an agarose gel. The 340 bp band (for single strand breaks) was isolated by electroelution into DEAE cellulose paper as described by Dretzen *et al.* (14).

The M13 hybridisation and/or ethanol precipitation step(s) is not efficient for DNA below 45 base pairs. Thus to elucidate bleomycin damage for fragments less than this length a more conventional technique was used. The 340 bp α -DNA was 3' labelled with [^{32}p] dATP and then restricted with EcoRI*. The recognition sequence for EcoRI* is AATT (10) which occurs once in 340 bp α -DNA. The uniquely labelled fragments 92 and 248 bp in length were isolated from a 4% native polyacrylamide gel. The 92 and 248 bp fragments were treated with bleomycin and run on sequencing gels. The bleomycin damage from bp 4 to 45 and 299 to 341 in figures 2 and 4 comes from these fragments.

M13 hybridisation reaction This method is fully described elsewhere (V. Murray and R. Martin - manuscript submitted for publication). Briefly [^{32}p] labelled α -DNA is hybridised to single stranded M13 DNA containing a cloned copy of α -DNA. α -M13 clones are used with either (+) or (-) strand α -DNA inserts. By this method either purified (-) or (+) strand α -DNA can be isolated. This technique also removes non- α -DNA sequences from the DNA preparation.

α -DNA recombinant DNA cloning The cloning of 340 bp α -DNA into M13 mp9 and pBR325 was carried out by conventional techniques and is described fully elsewhere (V. Murray and R. Martin - manuscript submitted for publication). The pBR325- α -B3 clone has a 340 bp α -DNA insert which has been fully sequenced and differs by 11 base substitutions from the "consensus" genomic α -DNA sequence.

RESULTS

Bleomycin damage with genomic 340 bp α -DNA The pattern of cleavage of purified genomic 340 bp α -DNA in vitro by 1 μM and 3 μM bleomycin is shown in figure 1. It can be seen that there is significant cleavage of the DNA above the undamaged control. There is a large variation in the intensity of

Figure 1 Autoradiograph of bleomycin damage on genomic and cloned 340 bp α -DNA. Lanes 1-6 depict (+) strand damage and lanes 7-12 (-) strand damage. Lanes 1-3 and 7-9 are genomic α -DNA and lanes 4-6 and 10-12 are cloned DNA. Lanes 2, 5, 8 and 11 have been treated with 1 μ M bleomycin, lanes 3, 6, 9 and 12-3 μ M bleomycin and lanes 1, 4, 7 and 10 - no bleomycin.

TABLE 1 DEGREE OF BLEOMYCIN DAMAGE AT DINUCLEOTIDE SEQUENCES

Dinucleotide	Total Sites	Very Strong Damage	Strong Damage	Medium Damage	Weak Damage	No Damage	% of Sites Cleaved
GT	20	8	6	4	2	-	100
GC	9	2	4	2	1	-	100
GA	23	-	1	9	8	5	78
GG	10	-	-	-	5	5	50
AT	17	-	1	2	6	8	53
AC	20	-	-	3	6	11	45
AA	31	-	-	2	7	22	29
AG	25	-	-	-	-	25	0
TT	32	-	-	-	5	27	16
TC	23	-	-	-	4	19	17
TA	16	-	-	-	2	14	12
TG	23	-	-	-	-	23	0
CT	25	-	-	-	3	2	12
CC	8	-	-	-	1	7	13
CA	23	-	-	-	4	19	17
CG	5	-	-	-	-	5	0

the bands in the gel. Damage hotspots have been assigned as being very strong, strong, medium or weak. With reference to densitometer scans of the autoradiographs of polyacrylamide gels, the ratios of the peak heights of very strong: strong: medium: weak damage are approximately 10:6:2:1. By using M13 α -DNA clones with either the (+) or the (-) strand, damage was surveyed over both strands of the 340 bp α -DNA.

When the location of the damage in the DNA sequence is determined by comparison with Maxam-Gilbert sequencing tracks (15), the sequence selectivity of the bleomycin can be elucidated. Certain dinucleotide sequences are preferred sites of cleavage (see Table 1) as found by other investigators (6-8). In particular 5' GT 3' and GC are invariably cleaved, GA is cleaved 78% of the time, and other sequences are cleaved less frequently.

Figure 2 The sequence specificity and intensity of bleomycin damage on both strands of genomic 340 bp α -DNA. One triangle represents weak damage, two triangles medium, three triangles strong, and four triangles very strong damage. From base pairs 4 to 156 on the (-) strand and base pairs 190 to 341 on the (+), damage was determined to the exact base pair; from base pairs 157-200 on the (-) strand and 151-189 on the (+) strand, damage was ± 1 base pair; base pairs 201-250 on the (-) strand and 101-150 on the (+) strand, damage was ± 2 base pairs; and base pairs 251-300 on the (-) strand and 50-100 on the (+) strand, damage was ± 3 base pairs.

In figure 2 the damage to both strands is presented and it can be seen that in most cases damage to one strand is not the same as damage on the opposite strand. In fact there are cases of very strong damage (bp 62, 197, 203, 261) where no damage is found on the opposite strand. Where there is a correspondence between cleavage on both strands it is usually at palindromic dinucleotides, eg. GC and AT.

Statistical analysis of DNA sequences treated with bleomycin Since bleomycin damage has been determined to the exact base pair for 310 base

TABLE 2 EFFECT OF THE BASE IMMEDIATELY 5' TO THE DINUCLEOTIDE ON DEGREE OF BLEOMYCIN DAMAGE

5' Base	I*		II*		III*	
	Observed	Expected	Observed	Expected	Observed	Expected
i) GT, GC, AT, AC dinucleotides						
G	1	3.9	4	2.7	5	3.4
A	3	8.1	5	3.7	13	7.2
T	19	8.9	2	6.2	2	7.9
C	4	6.2	8	4.3	4	5.5
$\chi^2_6 = 34.5$ $P < 0.00001$ (highly significant)						
ii) GA, GG, AA dinucleotides						
G	2	3.2	4	5.3	11	8.5
A	4	4.5	10	7.5	10	12.0
T	5	2.3	3	3.8	4	6.0
C	1	2.1	3	3.4	7	5.5
$\chi^2_6 = 7.9$ $P > 0.05$ (not significant)						
iii) all dinucleotides						
G	3	7.9	8	7.9	16	11.3
A	7	13.1	15	13.1	23	18.8
T	24	10.2	5	10.2	6	14.6
C	5	7.9	11	7.9	11	11.3
$\chi^2_6 = 37.8$ $P < 0.000005$ (highly significant)						

* Class I is strong GT and GC damage, strong and medium GA and AT, and medium AC and AA damage. Class II is medium GT and GC damage and weak GA, GG, AT, AC and AA damage. Class III is weak GT and GC damage and no GA, GG, AT, AC and AA damage. Expected values are calculated on the basis of their being no bias for any particular sequence or class.

pairs of DNA (see figure 2), the neighbour base sequences to the dinucleotide were investigated statistically to see what influence they had on the intensity of damage. The seven dinucleotides with significant bleomycin damage (GC, GT, AC, AT, GA, GG, AA) were divided into three categories according to relative intensity of damage. For example, GC site damage was assigned as being Strong (I), Medium (II) or Weak (III) damage, and AC site damage into Medium (I), Weak (II) and No Damage (III). Thus the influence of neighbouring sequences on the intensity of damage can be compared. The effect of G, A, T or C being present at the base immediately 5' to the dinucleotide (Table 2), the second base 5' to the dinucleotide, the third

Figure 3 Autoradiograph of bleomycin damage on genomic and cloned 92 bp *EcoRI** fragment of α -DNA. Lanes 1-3 are genomic and lanes 4-6 are cloned α -DNA. Lanes 2 and 5 have been treated with 1 μ M bleomycin, lanes 3 and 6 - 3 μ M bleomycin, lanes 1 and 4 - no bleomycin; lane 7 is a Maxam-Gilbert G+A sequencing reaction.

base 5', the first base 3' to the dinucleotide, the second base 3', or the third base 3' to the dinucleotide was determined. The data was analysed separately for the purine-pyrimidine dinucleotides (GC, GT, AC, AT), the

purine-purine dinucleotides (GC, GG, AA) as well as for all seven dinucleotides. The χ^2 values for each of the six positions next to the dinucleotide were less than 12.6 and therefore not significant ($P > 0.05$) except for the base immediately 5' to the dinucleotide. In this case (see table 2) the χ^2 value was only significant for the purine-pyrimidine and not the purine-purine dinucleotides. Inspection of the observed and expected values for the purine-pyrimidines reveals that T immediately 5' increases the intensity and A decreases the intensity of the damage. There is a suggestion that G also decreases the intensity of the damage.

Bleomycin cleavage with cloned and genomic 340 bp α -DNA The purified 340 bp α -DNA insert in pBR325 (the clone designated α B3) was damaged by bleomycin and compared with the damage in genomic 340 bp α -DNA. As shown in figures 1, 3 and 4, there are a number of differences between the cleavage patterns. For example compared to the genomic DNA, the cloned DNA damage has increased in intensity at base pairs 75, 85, 268 and 285, and decreased at base pairs 98, 163, 270 and 294. A total of 28 increases and 21 decreases in intensity were detected. The magnitude of the differences were measured by microdensitometry of the autoradiograph. The largest increases in intensity were at base pairs 283 and 285 where there was a 25 and 18 fold increase respectively. The largest decreases occurred at base pairs 55 and 91 where a 12 and 10 fold decrease (respectively) occurred.

In figure 4 bleomycin damage to genomic and cloned α -DNA is shown with reference to the DNA sequence. Some of the differences can be explained by base substitutions at the cleavage site, eg. at base pair 12 the strong hotspot at GT is changed to a weak hotspot at GA in the cloned sequence. Similarly base substitutions can explain the differences at base pairs 9, 186 and 335. However, most of the differences are more than 5 base pairs from the base substitutions. In fact the differences at base pairs 75 and 242 are at least 50 base pairs from the nearest base substitution. Thus some other mechanism must be invoked to explain the differences (see discussion).

In the cloned sequence the adenine at base pairs 53 and 54 is modified. It is probably modified to N⁶-methyladenine since T is present on the opposite strands, and A in the GATC sequence is N⁶-methylated in dam⁺ strains (in which the plasmid was grown). The G N⁶meA dinucleotide is not cleaved at base pair 54 in the cloned 340 bp α -DNA while weak damage is present in the genomic 340 bp α -DNA. Thus it appears that the presence of the methyl group on adenine makes it refractory to bleomycin cleavage.

Figure 4 Comparison of the sequence specificity and intensity of bleomycin damage. Solid triangles above the DNA sequence are for genomic α -DNA and open triangles below the line are for cloned α -DNA. More triangles indicate more damage (see legend to figure 2). A - (-) strand from base pairs 1-200. B - (+) strand from base pairs 151-344.

Single strand bleomycin damage with total human DNA and isolated 340 bp α -DNA

The M13 hybridisation technique also allows bleomycin damage to be assessed in total human DNA. Purified total human DNA was treated with bleomycin, cut with EcoRI and run on an agarose gel. As a control total human DNA previously cut with EcoRI was treated with bleomycin. After

agarose gel electrophoresis the 340 bp α -DNA band was isolated (to assess single strand break damage), 3'-labelled with [^{32}P] dATP, hybridised to M13 and the purified (-) strands were run on a sequencing gel. Isolated 340 bp α -DNA 3'-labelled before and after bleomycin damage was also run on the same gel. In all four cases the cleavage patterns were exactly the same in size and intensity (data not shown). This indicates that bleomycin damage in 340 bp α -DNA sequences in total human DNA and isolated 340 bp α -DNA is very similar.

DISCUSSION

By use of a novel system for separating the strands of 340 bp α -DNA, bleomycin damage could be assessed over a large part of the molecule. The M13 hybridisation system - whose characteristics are described in detail in another paper (V. Murray and R. Martin - manuscript submitted for publication) - separates the strands with very little cross contamination.

The bleomycin cleavage sites are associated with particular dinucleotide sequences (see table 1) as found by other workers (6-8). The dinucleotides GT and GC are cleaved all the time, GA most of the time and AA, GG, AC and AT were cleaved about 50% of the time.

Because a larger sequence was analysed for bleomycin damage - 310 bp were analysed where cleavage could be assigned to an exact nucleotide - statistical analysis of sequences next to the dinucleotide could be performed. It was found that only the first base 5' to a purine-pyrimidine dinucleotide influenced the intensity of damage caused by bleomycin; T in this position increased and A decreased the degree of cleavage. This effect has not been documented in a statistically significant manner before (although qualitative inspection of the data of Kross et al. (7) is suggestive of this trend with the dinucleotide AT) and the molecular basis by which it operates is not clear. It is possible that microvariation in the structure of the DNA helix (as discussed later) could be influenced in a particular manner by the first base 5' to the dinucleotide. The other five positions examined (second and third base 5' and first, second and third base 3' to the dinucleotide) and the first base 5' to a purine-purine dinucleotide (table 2) did not have a statistically significant effect on the degree of cleavage. Perhaps a survey of a larger number of base pairs damaged by bleomycin might reveal more subtle effects of neighbouring bases.

The most intense site for bleomycin cleavage is between base pairs 194 and 203. This is a region which contains ten bases with an alternating

purine-pyrimidine structure. In fact all 10 very strong damage sites occur in regions with four or more alternating purine-pyrimidines. Alternating purine-pyrimidine sequences are known to adopt a left-handed DNA structure under particular conditions. Z-DNA has been postulated to play an important role in the control of gene expression (16) and it is tempting to speculate that the specific cleavage of DNA sequences that can adopt Z-DNA conformations could be a component of the effectiveness of bleomycin as a cancer chemotherapeutic agent.

In figure 2 the position of single strand breaks on both strands of the 340 bp α -DNA molecule is presented. It is noticeable that there is very little correlation between the damage on one strand and that at the same position on the opposite strand except for the palindromic dinucleotides GC and AT. This lack of correlation highlights the stringency of the steric relationships between sites of binding and cleavage; presumably the Fe^{2+} is located nearer to one of the two strands and the other strand is out of effective range of the free radicals generated as a consequence of Fe^{2+} oxidation.

Analysis of single strand bleomycin damage with total human DNA and isolated 340 bp α -DNA indicated no significant differences. In the human genome α -DNA is mainly present as tandem 340 bp repeats and thus long range effects of a neighbouring α -DNA sequence were not detected.

The most surprising result from the experiments described in this paper was the differences in bleomycin damage between genomic 340 bp α -DNA and the cloned 340 bp α -DNA. There are forty-nine detectable differences in intensity between the bleomycin damage in the two DNAs. The largest differences represent about a ten to twenty-five fold change in intensity. There are eleven base substitutions in the cloned α -B3 DNA compared to the genomic "consensus" sequence but only four of the differences in bleomycin damage can be directly attributed to the base substitutions. Thus the other forty-five differences are not at the base substitution site and in the most extreme case one difference (base pair 71 - weak to medium) is fifty-seven base pairs from the nearest base substitution. The large difference (weak to strong) at base pair 85 is 43 base pairs from the nearest base substitution. If trivial explanations for this effect can be eliminated, then these long range effects are very interesting.

There are three classes of explanation for the differences between the genomic and the cloned α -DNA bleomycin damage. Firstly, the phenomenon can be explained by microvariation in the DNA structure and this is the

explanation that we favour. The DNA helix is not perfectly regular but can tolerate small changes in bond angles, propellor twist, etc. (17). Base substitutions could be able to alter these small changes in DNA structure over large distances in the DNA. If the binding and/or cleavage of DNA by bleomycin is sensitive to these small changes in DNA structure, then the observed differences in bleomycin damage caused by base substitution would be explained accordingly. There are some intriguing sequence-dependant parameters in the dodecamer DNA structure determined by X-ray diffraction by Dickerson and Drew (18):- purine-pyrimidine dinucleotides open their base planes toward the major groove, pyrimidine-purines open towards the minor groove, while pyrimidine-pyrimidines and purine-purines do not favour the major or minor groove; there are also sequence dependant changes in helical twist angles and propellor twist. Presumably such microvariation also occurs in longer DNA sequences and it seems possible that these (or other) structural variations could be a factor in the sequence specificity of bleomycin damage, changes in intensity of damage at various sites, and the long range effects of base substitutions.

Secondly, procedural artifacts could be responsible. The bacteria in which the plasmid was grown, methylate at GATC sequences and thus at positions 53 and 54 the adenine is N⁶-methylated in the cloned sequence and is not in the genomic sequence. In mammalian cells CG sequences are occasionally methylated, but of the 5 CGs present no 5-methylcytosine has been detected by Maxam-Gilbert sequencing (12). Thus methylation cannot by itself fully explain all the differences between the genomic and cloned α -DNA bleomycin damage but could contribute to the microvariation in DNA structure described above. Contaminating substances due to isolating the α -DNA from bacteria or mammalian cells cannot be ruled out but are very unlikely because the final steps in purification are the same, i.e. agarose gel electrophoresis, followed by electroelution of the 340 bp band.

Thirdly, microheterogeneity in the genomic α -DNA sequence can be considered as a possible explanation but can be ruled out on the basis of the magnitude of the changes observed. Variations (base substitutions, deletions and additions) amongst the multiple copies of the α -DNA sequence in the African Green Monkey genome are approximately 4%/nucleotide (13). Human α -DNA appears to be similar since the α -B3 clone had a 3% base substitution rate and preliminary sequence data of M13 α -DNA clones indicates a 4% variation. Variations above 10% should be picked up on Maxam-Gilbert sequencing gels and have not been detected (12) but even if

variation were 20%, the frequency of a "consensus" dinucleotide being present would be $(0.8)^2 = 0.64$. Thus with respect to increases in intensity of damage in the cloned material compared to the genomic sequence, $\frac{1}{0.64} = 1.6$ microheterogeneity can only account (in this extreme case) for a 1.6 fold increase. The changes observed (up to 25 fold) are obviously greater than this and therefore microheterogeneity in the DNA sequence cannot explain the phenomenon. However, decreases can be explained by microheterogeneity. For example, if a particular dinucleotide CC has no damage associated with it in the cloned DNA but in the genomic DNA a small proportion of strong damage GC is present, then a no damage site could be converted to a weak damage site. However, the number of decreases in damage intensity which are caused by microheterogeneity is not expected to be large because of the combination of circumstances necessary for their occurrence, and also it is probably only significant in no damage to weak or medium damage changes - 10 of the 21 decreases.

We are currently extending this work to look at bleomycin single and double strand break damage in intact cells.

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