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DNA Damage and Growth Inhibition in Cultured Human Cells by Bleomycin Congeners†

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ABSTRACT: Bleomycin is hypothesized to cause cell growth inhibition and cell death via DNA cleavage. We have attempted to determine if net DNA cleavage is directly related to growth inhibition by measuring whether both parameters vary in parallel. Of primary importance to these studies was use of several bleomycin congeners. We have shown that these congeners vary in their abilities both to inhibit cell growth and to cause DNA damage. Bleomycin B_2, tallysomycin, and phleomycin were the most potent growth inhibitors, and bleomycin B_2 caused the most DNA damage. *N*-Acetylbleomycin A_2 was inactive in both assays. The net amount of DNA damage measured at two levels of growth inhibition was compared for each congener and was found to vary widely among the congeners. Similarly, the degree of growth inhibition at a given level of submaximal DNA damage was found to vary widely when individual congeners were compared to each other. Hence, growth inhibition and net DNA damage due to bleomycin are not directly correlated with each other when individual congeners are compared to each other.

The bleomycins are glycopeptide-derived antitumor antibiotics isolated from cultures of *Streptomyces verticillus* (Umezawa et al., 1966). Blenoxane, a mixture of bleomycin A_2 (60–65%), bleomycin B_2 (~30%), and several other bleomycins, including demethylbleomycin A_2 (Crooke & Bradner, 1976), is used clinically for the treatment of certain tumors (Carter et al., 1978; Umezawa et al., 1972; Umezawa, 1976). Additional bleomycin group antibiotics investigated as potential antitumor agents include the phleomycins (Bradner & Pindell, 1962; Umezawa et al., 1962) and tallysomycins (Kawaguchi et al., 1977; Konishi et al., 1977). Bleomycins, phleomycins, and tallysomycins are all known to induce breakage of chromosomal DNA (Kross et al., 1982b). Strand scission occurs both in vitro (D'Andrea & Haseltine, 1978; Sugiura & Suzuki, 1982; Suzuki et al., 1969) and in vivo (Iqbal et al., 1976; Suzuki et al., 1969; Terasima et al., 1970), and both single-strand and double-strand breaks are observed (Haidle, 1971; Suzuki et al., 1969; Terasima et al., 1970). Breakage is sequence selective (D'Andrea & Haseltine, 1978), but preferred cleavage sites differ somewhat among the three structural families (Kross et al., 1982b; Mirabelli et al., 1979, 1980, 1982a,b); the precise mechanism of DNA strand scission remains to be established.

Bleomycin-induced DNA scission is hypothesized to cause inhibition of cell growth (Barlogie et al., 1976; Barranco & Humphrey, 1971; Hittelman & Rao, 1974) and to cause cell death (Clarkson & Humphrey, 1976). Cells are accumulated in late G_2 phase of the cell cycle when treated with bleomycin under conditions which also cause DNA breakage (Burger et al., 1982; Hittelman & Rao, 1974). Inhibition in mitosis has also been observed (Tobey, 1972), and recovery from inhibition is very slow. Bleomycin also produces a dose-dependent decrease in colony-forming ability (Terasima et al., 1972). HeLa S_3 cells were less sensitive than L5 cells (Terasima & Umezawa, 1970), and the Cu(II) complex was more active against Ehrlich ascites cells than was metal-free bleomycin; metal-free bleomycin was more active than the Zn(II) or Fe(II) complexes (Rao et al., 1980).

A direct causative relationship between bleomycin-induced DNA damage and growth inhibition or cell death has not been conclusively established. This is true in most cases because carefully controlled experiments have not been performed to measure both parameters simultaneously. We have undertaken to do so in order to establish some basic correlations between bleomycin A_2 (BLM A_2)[1] induced growth inhibition and DNA strand breakage in cultured human cells. Additionally, to obtain information that may bear relevance to structural features of bleomycins that contribute to their antineoplastic

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[1] Abbreviations: demethyl-BLM A_2, demethylbleomycin A_2; BLM A_2, bleomycin A_2; BLM B_2, bleomycin B_2; iso-BLM A_2, isobleomycin A_2; epi-BLM A_2, epibleomycin A_2; *N*-Ac-BLM A_2, *N*-acetylbleomycin A_2; PLM, phleomycin; TLM, tallysomycin; EBSS, Earle's basal salt solution; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

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activity, we have carried out the first comparisons in human cells of the biological activities, i.e., growth inhibition and DNA breakage, of several bleomycin congeners.

Materials and Methods

Blenoxane, phleomycin, and tallysomycin were obtained from Bristol Laboratories through the courtesy of Dr. William Bradner. Blenoxane was fractionated as described (Oppenheimer et al., 1979; Chien et al., 1977) to provide bleomycin A_2, bleomycin B_2, and demethylbleomycin A_2. Bleomycinic acid (Tanaka & Takita, 1979) isobleomycin A_2 (Nakayama et al., 1973), epibleomycin A_2 (Kunishima et al., 1976), and *N*-acetylbleomycin A_2 (Oppenheimer et al., 1980) were prepared as described. Transformed human KB cells were obtained from Dr. Erling M. Jensen, E.G.R.G. Mason Research Institute, Worchester, MA. [^{3}H]Thymidine (67 Ci/mmol) and [^{14}C]thymidine (53–58 Ci/mmol) were obtained from ICN Pharmaceuticals, Irvine, CA. All tissue culture media and supplies were purchased from Flow Laboratories, McLean, VA, and one lot of fetal bovine serum was used in all studies. [^{3}H]DNA markers, λ phage and SV40 component I, were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, MD. Scintillation fluors, *N*-lauroylsarcosine, and sucrose (grade I) were obtained from Sigma Chemical Co.

Cell Culture. KB cells were maintained in monolayer culture in Eagle's basal medium with 10% fetal bovine serum at 37 °C in 5% CO_2. No antibiotics were used. Cells were routinely subcultured at one-fifth confluent density in 75 cm^2 plastic flasks (Falcon).

Staging and Treatment of Cell Cultures. A confluent 75 cm^2 flask of cells was subcultured at one-fifth density in two 75 cm^2 flasks. [^{3}H]thymidine (in studies of repair of DNA breaks) or [^{14}C]thymidine was added to give 0.1 μCi/mL in a final volume of 10 mL. After incubation for 24 h, the medium was removed, the cells were carefully rinsed with EBSS, and fresh whole medium (without radiolabel) was added. After incubation for 24–28 h, both flasks were trypsinized. Cells from one culture were divided into six 25 cm^2 plastic flasks while cells from the other culture were counted and plated at 2.5×10^5 cells mL^{-1} well^{-1} in 24-well multiwell dishes (Linbro). Twenty-four hours later, cells were treated with individual bleomycin congeners. This protocol allowed for simultaneous and identical treatment of cells for assay of both growth inhibition and DNA strand scission.

Sucrose Gradient Centrifugation. Alkaline sucrose density centrifugation was performed according to the method of Friedman et al. (1975). This method is designed to avoid shear and degradation of DNA so that a chromosomal-sized DNA complex is observable. The sedimentation rate of the complex is extremely sensitive to single-strand breaks (Ormerod & Lehman, 1971). Linear gradients (10 mL) of 5–20% sucrose containing 0.1 N NaOH, 0.9 M NaCl, and 10 mM EDTA were formed by displacement in polyallomer tubes. A 1.7-mL cushion of 60% sucrose containing 0.1 N NaOH was placed under each gradient. On each was layered 0.5 mL of lysing solution containing 0.45 N NaOH, 0.55 M NaCl, 10 mM EDTA, and 1% *N*-lauroylsarcosine. Treated cells were washed thoroughly with EBSS and resuspended in 2 mL of 10 mM Tris buffer (pH 7.4) containing 0.15 M NaCl and 1 mM EDTA. An aliquot of this suspension (0.2 mL, approximately 4.5×10^5 cells) was pipetted gently onto the lysing solution. All procedures were performed at 4 °C with minimal exposure of the cells to light. Lysis was continued overnight at 0 °C in the dark.

Gradients were centrifuged at 0 °C in a Beckman L8-70 ultracentrifuge using a Beckman SW41 rotor for 3 h at 17000 rpm (36000*g*). Also, in some experiments with BLM B_2, gradients were centrifuged for 340 min at 17000 rpm. Immediately following centrifugation, gradients were fractionated dropwise from the bottoms of the tubes and applied to Schleicher & Schuell GF/A glass fiber squares. After the squares were dried, they were washed (4 °C) twice in 5% trichloroacetic acid for 1 h and twice in 70% ethanol for 0.5 h. The squares were dried and counted in a toluene fluor containing 4 g/L PPO and 0.05 g/L POPOP. Results are expressed as the percent of total cpm for each gradient. Each gradient contained 2000–5000 cpm, and recovery from the gradients was approximately 98%.

Cells labeled with either [^{3}H]thymidine or [^{14}C]thymidine, but not treated with bleomycin, were analyzed for DNA size. Tritium-labeled cells showed slightly more DNA breakage than ^{14}C-labeled cells (data not shown), but breakage in either case was negligible compared to bleomycin-induced breakage.

Determination of Sedimentation Coefficients and Molecular Weights. Gradients were calibrated with λ phage DNA and SV40 component I DNA, having sedimentation coefficients in alkaline sucrose gradients of 40 S (Daniels et al., 1980) and 53 S (Reddy et al., 1978), respectively. Sedimentation coefficients of radiolabeled DNAs were estimated by linear interpolation according to the method of Martin & Ames (1961). Approximate molecular weights were determined by using Studier's equation: $s = 0.0528M^{0.400}$, where s is the sedimentation coefficient and M is the molecular weight (Studier, 1965).

Calculation of DNA Breaks. The average number of breaks in DNA was calculated from the term $M_C/M_E - 1$ where M_C is the weight-average molecular weight of untreated (control) cells and M_E is the modal weight-average molecular weight (i.e., the molecular weight calculated at the modal position of total cpm on the gradient) of bleomycin-treated cells (Cleaver, 1975). Under the conditions used for sedimentation, untreated cells yielded a DNA band of approximately 500 S, as expected (Berry & Collins, 1980; Elkind, 1971; Friedman et al., 1975). This represents the maximum size of intact chromosomal DNA, according to extrapolation of the calculations of Davison (1966). Smaller DNA bands satisfied the criteria for random distribution (Lehman & Ormerod, 1970; Ormerod & Lehman, 1971).[2] For simplicity of expression, DNA breaks are given relative to 10^8 bases.

Colony Formation Assay. KB cells were plated at half confluent density and allowed to attach overnight. After treatment with bleomycin for 24 h, the cultures were thoroughly rinsed, trypsinized, and plated in 60-mm petri dishes for assay of colony formation, according to the method of Ham & Puck (1962). Replicates of three to six dishes were incubated for 11 days, cultures were stained, and colonies (greater than 50 cells) were counted. Results are expressed as the percent of colonies obtained from control (untreated) cultures ("mean ± SE").

Assay of Growth Inhibition. Bleomycin-treated cells were rinsed thoroughly with EBSS and trypsinized. Cell suspensions were diluted with trypan blue, and cells were counted on a

[2] The chromosomal complex (approximately 500 S) sediments anomalously, possibly due to the presence of lipid (Elkind & Chang Liu, 1972). Smaller DNA molecules should sediment in a random distribution. We confirmed that the distribution of DNA from untreated cells was random in our hands by sedimenting cells after various pulse lengths and pulse chases, centrifuging after loading various amounts of cells onto gradients, centrifuging after loading various concentrations (in loading buffer) of cells onto gradients, varying centrifugation speed, varying centrifugation length, and admixing exogenous, purified marker DNA before centrifugation (Berry & Collins, 1981; D. E. Berry, unpublished results).

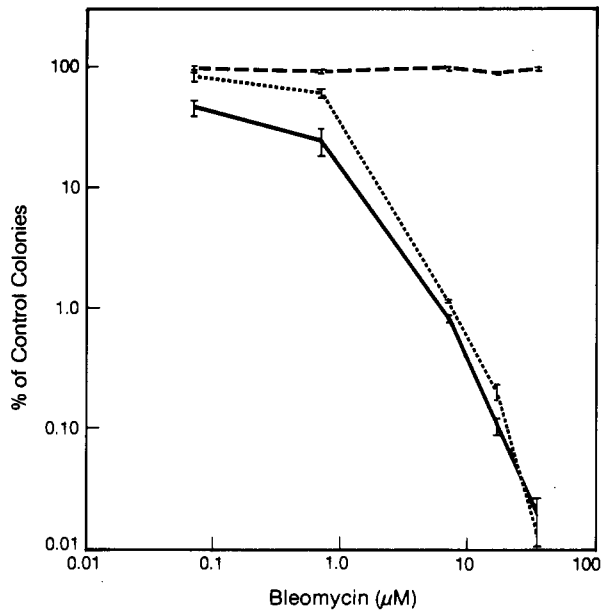

FIGURE 1: Colony formation by bleomycin-treated cells. KB cells were treated with bleomycin for 24 h and then plated for assay of colony formation, as described under Materials and Methods. Results are expressed as percent of untreated control cells (mean ± SE): BLM A_2 (—); BLM B_2 (···); *N*-Ac-BLM A_2 (- - -).

hemacytometer to determine the number of cells remaining and their percent viability. Results are expressed as mean ± SE of at least four determinations.

RESULTS

Colony Formation by Bleomycin-Treated Cells. KB cells were treated with BLM A_2 for 24 h and then assayed for their ability to form colonies according to the method of Ham & Puck (1962). For comparison, BLM B_2 and *N*-Ac-BLM A_2 were also assayed. The results (Figure 1) clearly indicated that BLM A_2 and BLM B_2, but not *N*-Ac-BLM A_2, decreased the viability of KB cells, as measured in this assay. BLM A_2 was slightly more potent than BLM B_2 at lower doses, but these two congeners were virtually equivalent at concentrations of 7.0 μM or more. *N*-Ac-BLM A_2, which does not cause growth inhibition or DNA breakage, was inactive.

Growth Inhibition by BLM A_2. KB cells exposed to as much as 207 μM BLM A_2 for 1 h did not exhibit loss of viability or decrease in number (Figure 2a). Cells exposed to BLM A_2 for 5 h showed no effect at concentrations up to approximately 70 μM. Viability (by trypan exclusion) was not affected up to 207 μM, but cell number was decreased at concentrations of 70 μM or more. This might reflect cells which have detached from the substratum and have lost viability, or cells which have detached within 5 h but remain viable and later reattach. In light of this observation, we did not attempt to relate growth inhibition to DNA breakage for cells treated with any bleomycin congener at a concentration greater than 35 μM.

KB cells were also exposed to BLM A_2 for periods ranging up to 72 h. The doubling time of these cells was determined to be 21 h (data not shown). Hence, if bleomycin-treated cultures were completely growth inhibited but not killed (i.e., if cell number remained unchanged from the time treatment was initiated), they would number 59%, 43%, 21%, and 9% of untreated cultures at 16, 24, 48, and 72 h, respectively. As shown in Figure 2b, inhibition was virtually complete after a 16-h exposure to 138 μM or more BLM A_2. Cells treated with 35 μM BLM A_2 were 80% of control. Cells treated for 72 h (Figure 2b) were not completely inhibited at any BLM

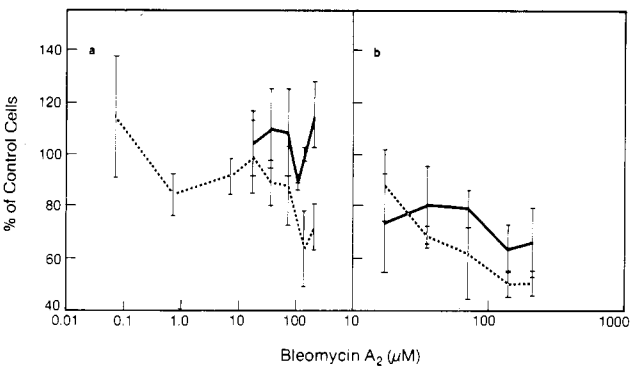

FIGURE 2: Growth inhibition by BLM A_2. KB cells were plated in multiwell dishes at 2.5×10^5 cells mL^{-1} $well^{-1}$ and incubated for 24 h. Fresh medium containing BLM A_2 was added, and after various times, cells were trypsinized, diluted with trypan blue, and counted. Results are expressed as percent of control (untreated) cultures (mean ± SE). Treated cultures would be 59% and 9% of control at 16 and 72 h, respectively, if completely inhibited. (a) 1 h (—), 5 h (···); (b) 16 h (—), 72 h (···).

A_2 concentration, implying recovery after an initial decrease in growth rate. Partial inhibition was observed with as little as 7 nM BLM A_2, and similar experiments with intermediate exposure times indicated that onset of growth inhibition was both dose and time dependent (data not shown). Viability (by trypan exclusion) was not affected by any treatment, and cell numbers were in no case decreased enough to indicate cell death (i.e., decreased below the number present at initiation of treatment).

Cells were also treated with BLM A_2, carefully washed free of the drug, and examined for recovery from growth inhibition during the following 72 h. Viability, by trypan blue exclusion, was not affected by any treatment. When cells were treated for 1 h, no culture was decreased below the level indicative of complete growth inhibition, and the time required for recovery from growth inhibition was dose dependent (data not shown). Also, when cells were treated for 24 h and allowed to recover for 24 or 48 h, growth inhibition was dose related (Figure 3). Complete growth inhibition was achieved only after exposure to at least 35 μM BLM A_2, resulting in cell numbers 19% and 12% of control at 24 and 48 h, respectively.

In summary, the data of Figures 1–3 indicated that exposure to BLM A_2 for 24 h was sufficient to cause marked inhibition of growth and to decrease cell viability, as determined by colony-forming ability. Continuous exposure for up to 72 h apparently caused growth inhibition but not immediate cell death. Cells treated for only 1 h were growth inhibited but eventually recovered, while cells treated for 24 h remained growth inhibited for at least another 48 h. While growth inhibition after a 24-h exposure did not appear to be directly related to decreased colony-forming ability in a quantitative sense, growth inhibition by any given bleomycin congener was found empirically to parallel the ability of that congener to mediate decreased colony-forming ability.

DNA Breakage by BLM A_2. In order to determine if growth inhibition was correlated with, and presumably caused by, BLM A_2 mediated DNA strand breakage, sister cells to those tested for growth inhibition were treated with BLM A_2, and their DNA was analyzed on alkaline sucrose gradients. DNA from untreated cells could be observed intact by the technique of lysis directly on gradients (Figure 4). Hence, this analysis is very sensitive, allowing reliable determination of as few as 0.15 break per 10^8 bases. Significant DNA breakage was observed in cells exposed to 0.07 μM or more BLM A_2 for 1 h (Table I); treatment with 35 μM BLM A_2 produced 1020

Table I: DNA Breakage by Bleomycin Congeners[a]

bleomycin derivative	DNA breaks/10^8 bases at a dose (μM) of					
	0	0.07	0.7	7	17	35
BLM A_2[b]	0	0.30 ± 0.4	1.16 ± 0.6	19 ± 2	[c]	1020 ± 59
BLM A_2	0	0	3.9 ± 0.5	9.9 ± 1	39 ± 5	45 ± 6
BLM B_2[d]	0	[c]	51.5 ± 8.5	141 ± 33	251 ± 42	369 ± 54
demethyl-BLM A_2	0	0	0.15 ± 0.008	3.6 ± 0.2	3.2 ± 0.2	4.0 ± 0.2
bleomycinic acid	0	0.63 ± 0.1	1.2 ± 0.2	1.2 ± 0.2	1.8 ± 0.3	8.8 ± 1
iso-BLM A_2	0	0	0.82 ± 0.09	0.82 ± 0.09	3.3 ± 0.4	3.3 ± 0.4
N-Ac-BLM A_2[e]	0	0	0	0.12	0	0

[a] Parental DNA of KB cells was labeled with [^{14}C]thymidine. Cells were treated with bleomycins at various doses for 24 h (and also for 1 h in the case of BLM A_2), washed, layered onto 5–20% alkaline sucrose gradients, lysed, and centrifuged at 36000*g* for 3 h. Gradients with BLM B_2 treated cells were centrifuged for 340 min in some experiments. Gradients were fractionated, and the percent of total cpm in each fraction was calculated. The number of breaks per 10^8 bases was determined as described under Materials and Methods and is expressed as mean ± SE. [b] One-hour treatment. [c] Not tested at this dose. [d] 127 ± 14 breaks per 10^8 bases at 3.5 μM. [e] Number of breaks shown is the highest observed rather than the mean.

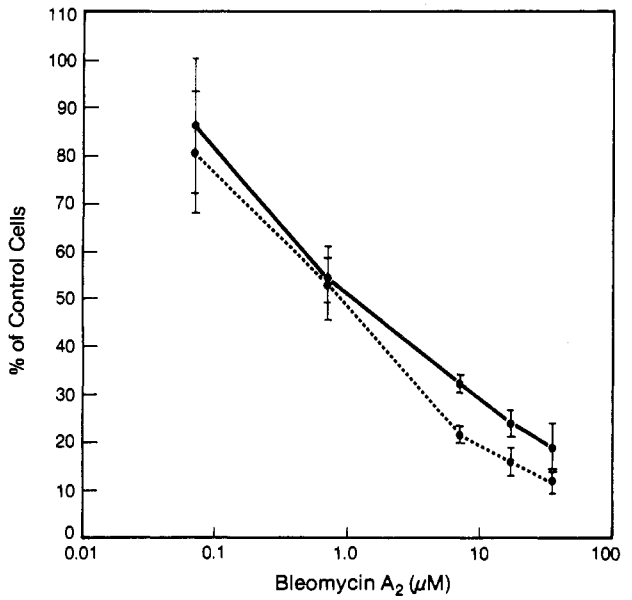

FIGURE 3: Recovery from growth inhibition by BLM A_2. KB cells were treated with BLM A_2 for 24 h. The medium was removed, the cells were thoroughly rinsed, and fresh, drug-free medium was added. Incubation was continued for 24 (—) or 48 h (···), and the cells were trypsinized and counted. Viability was assessed by trypan blue exclusion and did not vary from control cells. Results are presented as percent of control (untreated) cells (mean ± SE). If completely inhibited throughout the experiment, treated cultures would be 21% of control after a 24-h treatment + 24-h recovery and 9% of control after a 24-h treatment + 48-h recovery.

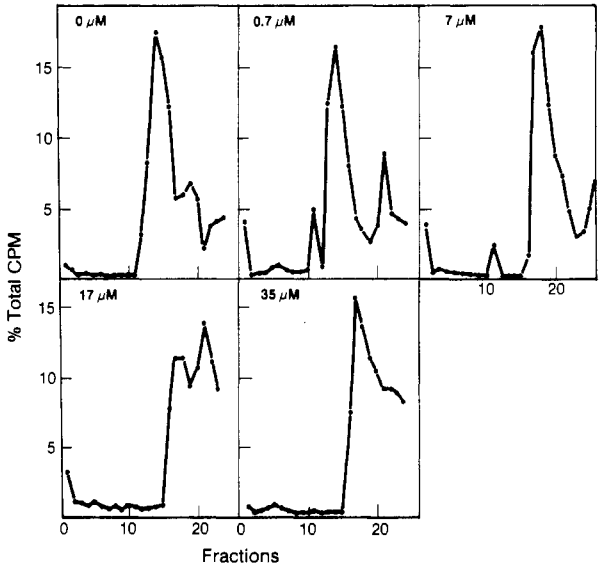

FIGURE 4: DNA breakage by BLM A_2 after 24 h. Parental DNA of KB cells was labeled with [^{14}C]thymidine. Cells were treated with BLM A_2 for 24 h, layered onto 5–20% alkaline sucrose gradients, lysed, and centrifuged for 3 h at 36000*g*. Gradients were fractionated from the bottom, and the percent of total radioactivity in each fraction was calculated. The number of breaks per 10^8 bases was determined as described under Materials and Methods. BLM A_2 concentrations are given with each gradient.

breaks per 10^8 bases. Strand scission was also present after exposure for 24 h (Figure 4), but the extent of cleavage was generally much less at each tested dose; e.g., 45 breaks per 10^8 bases were measured at 35 μM. These studies indicated that DNA breakage and cell growth inhibition occurred simultaneously in KB cells and that the extent of each was dose dependent. Since the relative number of breaks was less after 24 h of exposure of BLM A_2 than after 1 h, these studies also implied that repair of BLM A_2 induced DNA strand scission proceeded at a rate in excess of additional DNA damage after 1 h of treatment.

Since growth inhibition after treatment with less than 35 μM BLM A_2 for 1 h was reversed by 24 h, sister KB cells were analyzed for their ability to repair DNA breaks within this time. As shown in Figure 5, no DNA breakage was observed after treatment with as much as 35 μM BLM A_2, followed by a recovery period of only 5 h. Cells treated for 1 h and analyzed after a 24-h recovery period also showed no residual DNA breakage (data not shown).

Growth Inhibition by Other Bleomycins. A number of bleomycin congeners were assayed for their ability to inhibit growth of KB cells and to cause DNA breakage. The information accumulated not only permitted comparison of the biological activities of individual congeners in intact human cells but also provided a further basis upon which to determine if bleomycin-induced cytokinetic effects are correlated with DNA strand scission. Exposure to each of the congeners was carried out for 24 h, based on initial experience with BLM A_2; it was anticipated that this protocol would facilitate the identification of congeners with greater or lesser potency than BLM A_2.

Results of growth inhibition assays are shown in Figure 6. Percent viability was not changed by any treatment and remained greater than 95%. In no case was growth inhibition greater than 100% (i.e., 43% of control), implying that cell kill did not occur. TLM and PLM were the most potent congeners, followed closely by BLM B_2. These three agents produced complete inhibition at 35 μM concentration. BLM A_2, demethyl-BLM A_2, and iso-BLM A_2 were equivalent to each other, while bleomycinic acid was weakly inhibitory and *N*-Ac-BLM A_2 was inactive.

DNA Breakage by Bleomycins. Sister cells to those analyzed for bleomycin-induced growth inhibition were analyzed

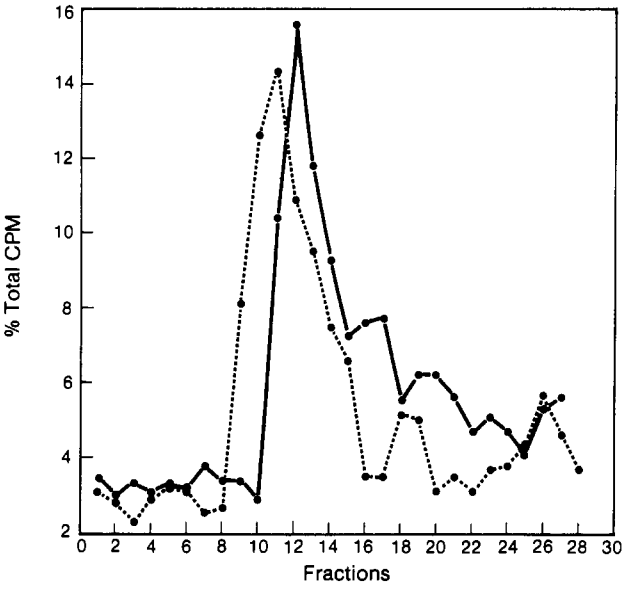

FIGURE 5: Repair of DNA breakage induced by BLM A_2. Parental DNA of KB cells was labeled with [^{14}C]thymidine, and cells were treated with BLM A_2 for 1 h. Medium containing BLM A_2 was removed from the cultures, cells were washed thoroughly with EBSS, fresh medium was added, and incubation was continued for 5 h. Cells were analyzed on alkaline sucrose gradients as described under Materials and Methods: untreated cells (—); cells treated with 35 μM BLM A_2 followed by a 5-h repair period (···).

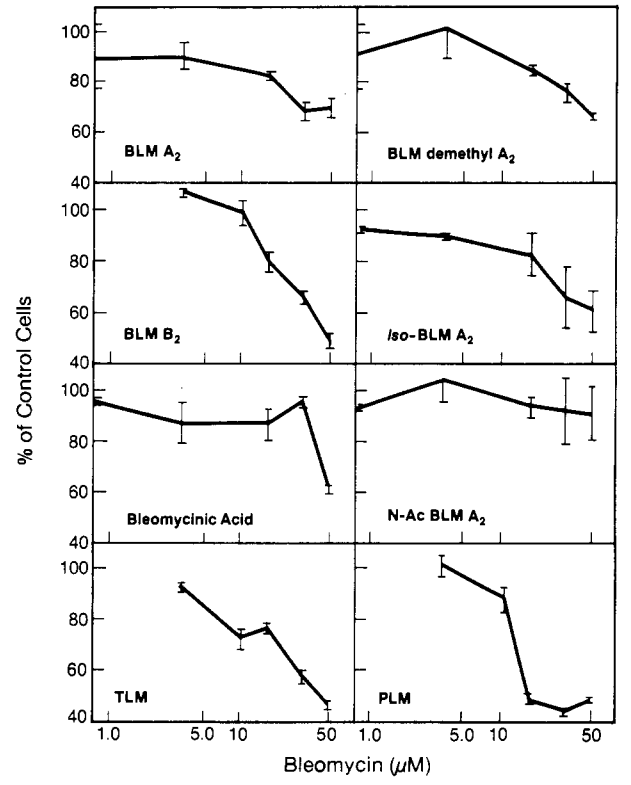

FIGURE 6: Growth inhibition by bleomycin analogues. KB cells were plated in multiwell dishes at 2.5×10^5 cells mL^{-1} well^{-1} and incubated for 24 h before drug addition. Cells exposed to BLM A_2, BLM B_2, demethyl-BLM A_2, iso-BLM A_2, bleomycinic acid, or *N*-Ac-BLM A_2 were sister cells to those analyzed for DNA breakage and contained [^{14}C]thymidine-labeled DNA. After 24 h, cells were trypsinized and diluted with trypan blue prior to counting. Results are expressed as the percent of those cells counted in untreated (control) cultures (mean ± SE).

for net DNA breakage on alkaline sucrose gradients. The data are summarized in Table I. BLM B_2 induced the highest number of breaks (369) after treatment at 35 μM concentration. Demethyl-BLM A_2 caused only 1% as much breakage at 35 μM. Bleomycinic acid was the only congener effective at a concentration as low as 0.07 μM, but breakage did not increase dramatically with increasing dose. iso-BLM A_2 was rather weak, while *N*-Ac-BLM A_2 was inactive.

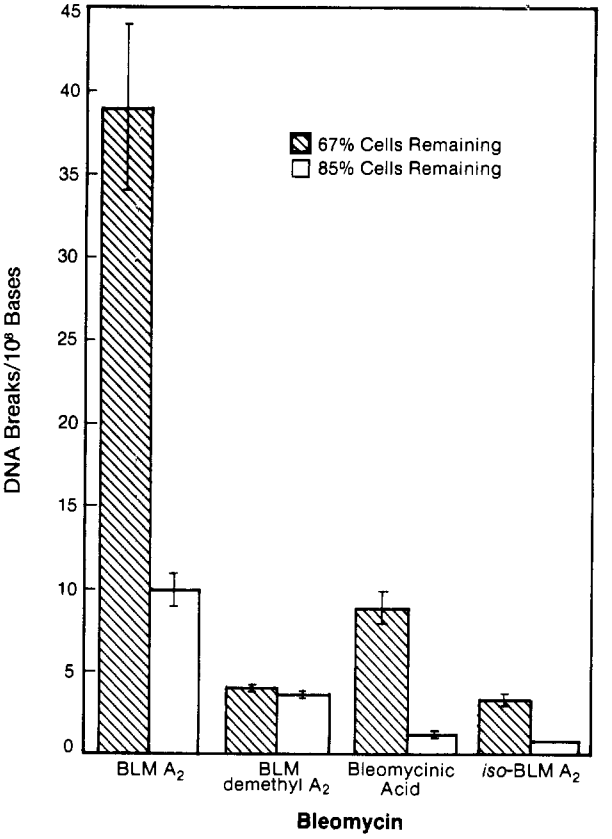

FIGURE 7: DNA breakage by bleomycin congeners at equivalent levels of growth inhibition. The number of DNA breaks per 10^8 bases (from Table I) was plotted for each bleomycin congener at two levels of growth inhibition (from Figure 6).

Correlation between DNA Strand Breaks and Cell Growth Inhibition. If DNA strand breakage by bleomycin were the cause of cellular growth inhibition, then it would be logical to assume that cells treated with different bleomycin congeners and suffering equal amounts of DNA breakage should be growth inhibited to the same extent. Similarly, it would be logical to assume the converse, i.e., that cells growth inhibited to the same extent by different bleomycin congeners should show equivalent amounts of DNA breakage. These comparisons were made for four bleomycin congeners, and the data are summarized in Figures 7 and 8. In Figure 7, the net amount of DNA breakage (from Table I) was plotted for each congener at two levels of growth inhibition: 85% and 67% of control cells remaining. The net amount of DNA breakage by different congeners was not equivalent at equal degrees of growth inhibition. In experiments where BLM A_2 treated cells were 67% of control, the net amount of DNA breakage was 4–10 times greater than DNA breakage in cells treated with other congeners and inhibited to 67% of control. The situation was similar when cells were inhibited to 85% of control. iso-BLM A_2, for instance, appeared to effectively inhibit cell growth when relatively little DNA breakage was measurable.

When the same congeners were compared for extent of growth inhibition at equal net amounts of DNA breakage (Figure 8), wide variation was again apparent. Treatments were compared in which net DNA breakage was 4 breaks per 10^8 bases; cells treated with either BLM A_2 or demethyl-BLM A_2 remained at higher densities than cells treated with iso-

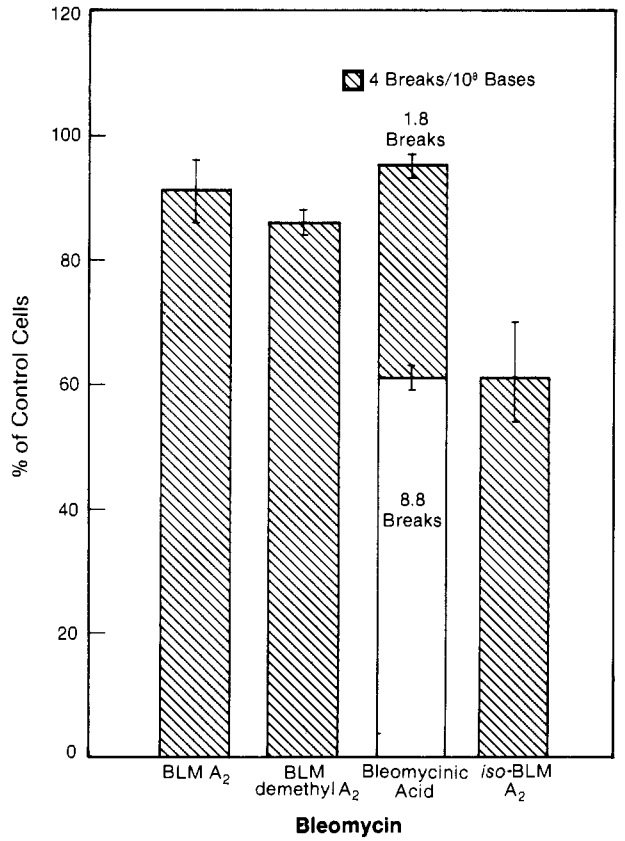

FIGURE 8: Growth inhibition by bleomycin congeners at equivalent levels of DNA breakage. Growth inhibition, as percent of control cells (from Figure 6), was plotted for each bleomycin congener at equivalent levels of DNA breakage. DNA breakage was 4 breaks per 10^8 bases for each of the congeners except bleomycinic acid. For bleomycinic acid, 1.8 and 8.8 breaks per 10^8 bases were measured at 95% and 61% of control cells, respectively.

BLM A_2. Again, Figure 8 indicated that iso-BLM A_2 inhibited cell growth effectively while relatively little DNA breakage was measurable. Cells treated with bleomycinic acid had twice as many DNA breaks when inhibited to the same degree as iso-BLM A_2 treated cells (8.8 vs. 4.0 breaks per 10^8 bases).

In summary, the data of Figures 7 and 8 showed that in KB cells treated for 24 h, the net amount of bleomycin-induced DNA damage varied widely among several congeners. Likewise, inhibition of KB cell growth varied widely among these congeners. More importantly, though, when these congeners were compared to each other with respect to these two parameters, no clear relationship between net DNA breakage and growth inhibition was evident.

DISCUSSION

While bleomycins are thought to inhibit cell growth and to decrease cell viability by virtue of their ability to break chromosomal DNA, evidence in support of this mechanism is inconclusive. We sought to determine if a clear relationship exists between the extent of DNA breakage and growth inhibition after bleomycin treatment. The protocols employed here facilitated this comparison by providing for the simultaneous analysis of both parameters in identically treated cells. On the assumption that these parallel effects should obtain for any bleomycin congener, regardless of absolute potency, several bleomycin congeners were compared for growth inhibition and net DNA strand scission.

Our results indicated that bleomycins decreased cell viability, as assessed by colony formation (Figure 1) and inhibited cell growth (Figures 2 and 6). Cells could recover from BLM A_2 induced growth inhibition if exposure was sufficiently short (e.g., 1 h) but not necessarily after exposure for 24 h (Figure 3). Continuous treatment for up to 72 h apparently did not result in cell death. BLM A_2 induced rapid dose-dependent breakage of chromosomal DNA (Figure 4), but DNA repair was also quite rapid, in both the absence (Figure 5) and presence of BLM A_2 (Table I, compare 1-h treatment to 24-h treatment). Various bleomycin congeners were examined for their ability to inhibit cell growth and to cause DNA breakage. A wide range of potencies was observed; *N*-Ac-BLM A_2 was completely inactive while BLM B_2 was much more active than BLM A_2 (Figure 6 and Table I). It was obvious, however, that while net DNA breakage and growth inhibition appeared to be dose related for each individual congener, the amount of net DNA breakage per se did not bear a direct relationship to the degree of growth inhibition (Figure 7), and vice versa (Figure 8), when these congeners were compared to each other.

We observed that growth inhibition by several bleomycin congeners was complete by 24 h (Figure 6). Treatment with >35 μM BLM A_2 caused a decrease in cell number at 5 h (Figure 2a), but in no other case was cell death apparent after continuous exposure. Also, high concentrations of BLM A_2 did not cause a decrease in cell number after 1 h (Figure 2a) or after treatment for 1 h and subsequent culturing in drug-free medium for up to 72 h (data not shown). Cells treated with 35 μM or more BLM A_2 for 1 h remained growth inhibited for at least 72 h, however. In light of the decrease in colony-forming ability shown in Figure 1, these data indicate that cell division after bleomycin treatment is probably required for loss of reproductive capacity to become manifest. Bleomycin causes a cell cycle block in G_2 (Barlogie et al., 1976; Barranco & Humphrey, 1971), and upon release of this block cells proceed into mitosis (Tobey, 1972). Aberrant mitosis as a consequence of bleomycin damage probably causes loss of viability in the daughter cells. This damage is thought to involve nuclear DNA, so that daughter cells do not receive intact, functional genomes and cannot complete a second round of DNA replication (Clarkson & Humphrey, 1976). DNA repair after BLM A_2 treatment was remarkably rapid, however. After a 1-h treatment, repair was complete within 5 h (Figure 5), and breakage accumulated over 24 h was decreased to approximately 5% of that incurred during the first hour (Table I). This implies that DNA repair begins immediately after initiation of DNA damage and that the net rate of repair not only equals but eventually exceeds the net rate of damage. It seems likely, therefore, that bleomycin-induced damage of specific genetic elements is more critical to cell viability than is the gross level of DNA damage.

Previous studies have indicated that cytotoxicity of bleomycin congeners is not correlated with their abilities to bind and cleave DNA (Oppenheimer et al., 1980; Asakura et al., 1975; Roy et al., 1981; Takahashi et al., 1979; Takeda et al., 1979). The abilities of BLM A_2, BLM B_2, and PLM to cleave DNA in a cell-free system are roughly equivalent while that of TLM is much less, even though TLM seems to bind DNA more tightly and at more sites (Kross et al., 1982b; Huang et al., 1980; Strong & Crooke, 1978). TLM has been shown to be more cytotoxic than BLM A_2 to microbial (Kawaguchi et al., 1977) and tumor (Carter et al., 1978) cells. We did not examine TLM for its ability to induce DNA strand breakage in KB cells, but we found PLM and TLM to be more potent growth inhibitors than BLM B_2 (Figure 6). Additionally, we found PLM to be at least as potent as BLM B_2 at causing DNA breakage in KB cells (data not shown), and

BLM B_2 was much more potent than BLM A_2 (Table I). It is reasonable to assume that once growth inhibition is complete, the amount of DNA breakage might bear no relationship to inhibition. However, even at intermediate degrees of growth inhibition, i.e., 67% or 85% of control, DNA breakage varied widely among several congeners (Figure 7). Similarly, as shown in Figure 8, the degree of growth inhibition varied among congeners at a given level of submaximal DNA breakage. The apparent lack of correlation between growth inhibition and net DNA breakage when bleomycin congeners were compared to each other might have arisen for several reasons, including the possibilities that (1) growth inhibition results, at least in part, from a biological effect other than DNA breakage, (2) the precise mechanisms (and hence possibly rates) of DNA breakage or repair vary slightly among congeners, (3) membrane permeability, and hence access to intracellular sites of inhibition, varies among congeners, or (4) damage of specific, critical DNA segments is responsible for growth inhibition.

In general, the data in this report confirm the structure–activity relationships proposed previously (Kross et al., 1982a,b; Oppenheimer et al., 1979; Takeda et al., 1979; Takeshita et al., 1981). In particular, the integrity of a positively charged C-terminal side chain is of primary importance in conferring DNA-cleaving potential upon a bleomycin congener, possibly by virtue of side chain interaction with negatively charged phosphate groups in the DNA. Demethyl-BLM A_2, which lacks the positively charged sulfonium moiety, and bleomycinic acid, which lacks the C-terminal amine moiety, caused less DNA strand scission than did BLM A_2 in cultured cells (Table I).

Interestingly, iso-BLM A_2 appeared to effectively inhibit cell growth while causing relatively little DNA breakage (Figure 7), and although the reason for this remains unclear, the observation might be of some interest from a therapeutic perspective. At the opposite extreme, and also of some relevance, is BLM B_2, which effectively inhibited cell growth but caused a very large amount of DNA damage.

Registry No. BLM A_2, 11116-31-7; BLM B_2, 9060-10-0; demethyl-BLM A_2, 41089-03-6; iso-BLM A_2, 51041-93-1; epi-BLM A_2, 62624-79-7; *N*-Ac-BLM A_2, 70772-32-6; bleomycinic acid, 37364-66-2; phleomycin, 11006-33-0; tallysomycin, 67995-68-0; bleomycin, 11056-06-7.

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DNA Damage Induced by Bleomycin in the Presence of Dibucaine Is Not Predictive of Cell Growth Inhibition†

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ABSTRACT: Growth inhibition and cell killing by bleomycin are believed to be related to the ability of this antibiotic to cleave chromosomal DNA. Because bleomycin has an intracellular site of action, its ability to cross biological membranes must be critical to its overall effectiveness as an antitumor agent. The local anesthetic dibucaine acts to enhance membrane fluidity; therefore, the reported ability of this local anesthetic to modulate bleomycin effects on KB cells was investigated. Cells were treated with various bleomycin congeners in the presence or absence of dibucaine for 24 h. Dibucaine enhanced the inhibition of cell growth mediated by bleomycin A_2, demethylbleomycin A_2, bleomycin B_2, and isobleomycin A_2. *N*-Acetylbleomycin A_2 did not inhibit cell growth in the absence of dibucaine, but it was inhibitory in the presence of dibucaine. Cells treated simultaneously for analysis of DNA breakage on alkaline sucrose gradients revealed that breakage was also enhanced in the presence of dibucaine. The degree of enhancement varied with dose and bleomycin congener. *N*-Acetylbleomycin A_2 did not induce DNA breakage in either the absence or the presence of dibucaine. While growth inhibition and net DNA breakage correlated reasonably well in the absence of dibucaine for each bleomycin analogue tested, proportionality was lost in the presence of dibucaine, and very little DNA breakage was present when growth inhibition was complete. These observations imply that, at least in the presence of dibucaine, bleomycin may mediate growth inhibition at some locus in addition to chromosomal DNA and, also, that a given net amount of bleomycin analogue induced DNA damage per se does not produce a specific degree of growth inhibition.

The antitumor antibiotic bleomycin is thought to inhibit cell growth and to cause cell death by virtue of its ability to cleave chromosomal DNA (Clarkson & Humphrey, 1976; Hittelman & Rao, 1974; Kross et al., 1982; Terasima et al., 1972; Terasima & Umezawa, 1970). Consistent with this scheme, Clarkson & Humphrey (1976) showed that treatment of Chinese hamster ovary (CHO)[1] cells with bleomycin for 30 min produced extensive DNA strand breakage and caused cells to accumulate in the G_2 phase of the cell cycle. Hittelman & Rao (1974) found that DNA damage, as assessed by premature chromatin condensation, was present in cells blocked in late G_2; others have also presented evidence that bleomycin induces a G_2 block (Barlogie et al., 1976; Barranco & Humphrey, 1971; Nagatsu et al., 1972; Watanabe et al., 1974), presumably due to DNA damage. When colony formation of bleomycin-treated cells was assayed, cell death appeared to be correlated with DNA breakage (Kohn & Ewig, 1976).

Several observations indicate that bleomycin cytotoxicity can be modulated by the state of the cell membrane. Pertinent

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[1] Abbreviations: CHO, Chinese hamster ovary; demethyl-BLM A_2, demethylbleomycin A_2; BLM A_2, bleomycin A_2; BLM B_2, bleomycin B_2; iso-BLM A_2, isobleomycin A_2; *N*-Ac-BLM A_2, *N*-acetylbleomycin A_2; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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