EFFECT OF BLEOMYCIN ON AN RNA-DNA HYBRID

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SUMMARY: The antitumor antibiotic bleomycin degrades the poly(dT) portion of a poly(rA)·poly(dT) hybrid formed by the action of reverse transcriptase. The poly(rA) moiety of the hybrid is not degraded by the drug. This provides firm evidence that the secondary structural differences between RNA and DNA are not responsible for the specificity of bleomycin toward DNA.

The antitumor drug, bleomycin, has been shown to cause the *in vitro* degradation of both single- and double-stranded DNAs, as well as synthetic deoxyribopolymers (1, 2). This degradation reaction involves the release of free bases from the DNA, damage to the deoxyribose moiety, and the rupture of the phosphodiester bonds, resulting in extensive fragmentation of the DNA (3, 4). The final product of the reaction consists of single-stranded oligonucleotides with a molecular weight of approximately 3,000 daltons; these oligonucleotides are resistant to further drug action (5). No comparable reaction can be demonstrated for RNA or synthetic ribopolymers (6), although RNA competes with DNA in the bleomycin reaction. It has been shown that the lack of reactivity towards RNA is not the result of the presence of uracil (or absence of thymine) in the RNA, since bacteriophage PBS-1 DNA, which contains deoxyuridine in place of thymidine, is extensively degraded. This degradation is accompanied by the release of free uracil and the other three bases (6). That the difference in reactivity is caused by the difference in secondary structure between RNA and DNA seems unlikely, since both double- and single-stranded DNAs are degraded; and in addition,

no reaction is observed with the double-stranded DNA-like complex poly(rA)·poly(rU) (6).

The present study was initiated to determine the bleomycin susceptibility of an RNA-DNA hybrid, to yield further information regarding the specificity of bleomycin attack.

MATERIALS AND METHODS:

The RNA-DNA hybrid used in this study was the product of the RNA-directed DNA polymerase (reverse transcriptase) reaction, using either a crude enzyme preparation from Rauscher murine leukemia virus (MuLV) or the purified avian myeloblastosis virus (AMV) enzyme, which was a generous gift from Dr. Joseph Beard, Life Sciences Research Labs., St. Petersburg, Florida, through the auspices of Dr. M. A. Chirigos of the National Cancer Institute. This AMV enzyme contained 29,316 units/mg protein; one unit of activity is defined as the amount of enzyme which will catalyze the incorporation of one nmole of dTMP into an acid-insoluble product in 10 min. at 37°C. The MuLV enzyme was prepared from mouse JLS-V5 cells, obtained through the courtesy of Dr. Patton T. Allen of this Institution. The JLS-V5 cells were grown in Eagle's Minimum Essential Medium with Earle's salts and 10% fetal calf serum. The virus-containing medium from these cells (about 500 ml) was clarified by centrifugation at 4,000 x g for 15 min. at 5°C. The supernatant fluid was centrifuged in the Beckman 30 rotor at 30,000 rpm for one hour at 5°C. The resulting pellets were resuspended by homogenization in 8 - 10 ml of Tris-NaCl buffer (0.15 M NaCl; 0.015 M Tris; pH 7.6) and layered onto 15 ml of 20% glycerol in the Tris-NaCl buffer. These tubes were centrifuged for two hours in the Beckman 30 rotor at 30,000 rpm and 5°C. The pellets were resuspended in approximately 1 ml of T-T-4 buffer (0.05 M Tris; 0.4% Triton X-100; pH 8.3) and stored at -70°C.

In both enzymatic reactions, the template was 48.0 μM [^{14}C]poly(rA) (0.77 μCi/μmole P; Miles Labs., Kankakee, Illinois); the primer was 1.875 μM $(dT)_{12-18}$ (P-L Biochemicals, Milwaukee, Wisc.); and the substrate was 1.0 μM [*methyl*-^{3}H] thymidine-5'-triphosphate ([^{3}H]dTTP; 52.6 mCi/μmole; New England Nuclear, Boston, Mass.). The MuLV reaction mixtures also contained: 50 mM Tris, pH 8.3; 5.0 mM dithiothreitol; 2.5 mM $MnCl_2$; 0.1 M NaCl; 0.2% Triton X-100. The AMV reaction mixtures also contained: 50 mM Tris, pH 8.3; 25 mM dithiothreitol; 6.0 mM $MgCl_2$; 15 mM KCl. Approximately 20 units of enzyme was used for the AMV mixtures. The amount of enzyme used for the crude MuLV system was not determined since it varied among the viral lysates used, but was approximately 1 unit.

Both enzymatic reactions were incubated for 1 - 2 hours at 37°C. The product of the reverse transcriptase reaction was reacted for one hour at 37°C with bleomycin (a gift of Bristol Laboratories, Syracuse, New York) and 2-mercaptoethanol as indicated in the legends to the figures. The final reaction mixture (1.0 ml) was mixed with 4.0 ml of Cs_2SO_4 to yield a final density of approximately 1.45 g/ml and centrifuged for 96 hours at 36,000 rpm and 20°C in the SW 50.1 rotor of a Beckman L2-65B ultracentrifuge.

After centrifugation, 10-drop fractions were collected from the top of the gradient, directly onto 2.3-cm Whatman 3 MM filter paper discs. The density across the gradient was determined by refractive index. Yeast RNA (0.2 mg) was added to each disc as carrier, and the discs were prepared for counting as previously described (2).

RESULTS:

Fig. 1 shows the effect of bleomycin on the RNA-DNA hybrid produced by the MuLV reverse transcriptase. This reaction employed [^{14}C]poly(rA) as the template, and incorporated [^{3}H]TTP into the poly(dT) strand of the hybrid. As can be seen from the figure, a portion of the radioactivity in the untreated sample (both ^{3}H and ^{14}C) corresponds approximately to the density expected for the hybrid molecule poly(rA)·poly(dT) (7). Some of the ^{14}C radioactivity of the control corresponds to the density of the poly(rA) template alone. In the case of the sample treated with bleomycin, virtually all of the ^{3}H radioactivity (poly(dT)) is removed from the gradient profile, while the ^{14}C radioactivity (poly(rA)) remains. Very similar results are observed in Fig. 2, in which the purified and more active AMV enzyme was used to form the hybrid. In this case the poly(rA) does not form two distinct

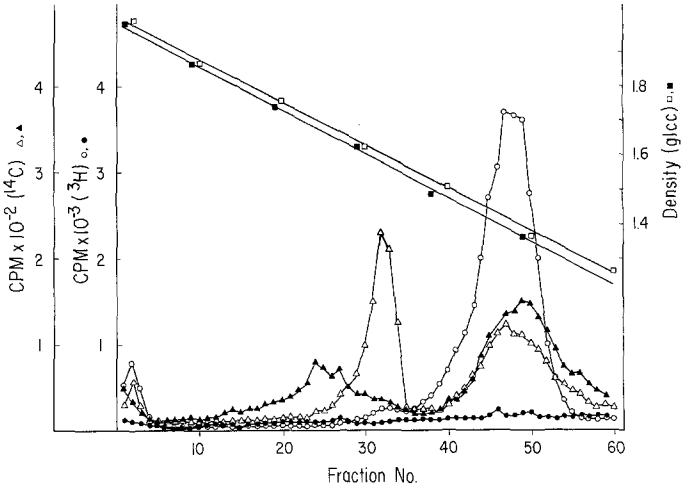

Figure 1: Action of bleomycin on MuLV reverse transcriptase product. Cs_2SO_4 equilibrium density-gradient profile. In this and all following figures, the product of the reverse transcriptase reaction was treated with 1.5 mg/ml of bleomycin (where indicated) and 20 mM 2-mercaptoethanol. All samples were incubated for 1 hour at 37°C prior to mixing with Cs_2SO_4 and centrifugation. The poly(rA) was labeled with ^{14}C; the poly(dT) (product) was labeled with ^{3}H. Sample #1, no bleomycin: ○, ^{3}H radioactivity; △, ^{14}C radioactivity; □, density. Sample #2, + bleomycin: ●, ^{3}H radioactivity; ▲, ^{14}C radioactivity; ■, density.

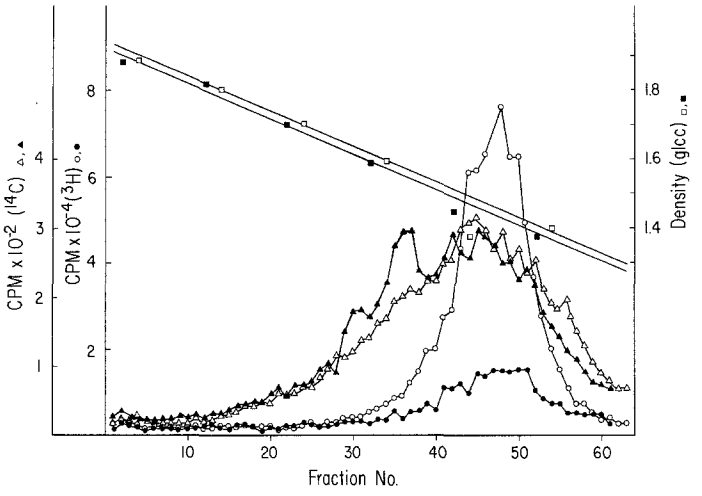

Figure 2: Action of bleomycin on AMV reverse transcriptase product. Cs_2SO_4 equilibrium density-gradient profile. The symbols are the same as for Fig. 1.

peaks in the control sample, but there is a peak of ^3H and ^{14}C radioactivity in the hybrid region of the gradient. Again, most of the ^3H radioactivity is removed from this peak after treatment with bleomycin. Figure 3 illustrates the effect of bleomycin on a similar AMV enzyme hybrid formed in the presence of 0.025 M NaF. Fluoride at this concentration causes approximately a 70% inhibition of the RNase H activity while inhibiting the DNA polymerase activity by less than 25% (8). RNase H degrades the RNA portion of the RNA-DNA hybrid and is thus far not separable from reverse transcriptase. Although there is not as much hybrid formed in the presence of fluoride (Fig. 3), it is still apparent that the bleomycin preferentially degrades the poly(dT) portion, leaving the poly(rA) intact. Finally, Fig. 4 shows the effect of bleomycin on the poly(rA) template. In this experiment, the AMV reverse transcriptase reaction was set up as before, except that the [^3H]TTP was omitted. It can be seen that the drug has no effect either on the amount or the buoyant density of the template.

DISCUSSION:

From the results presented, it is quite clear that bleomycin possesses

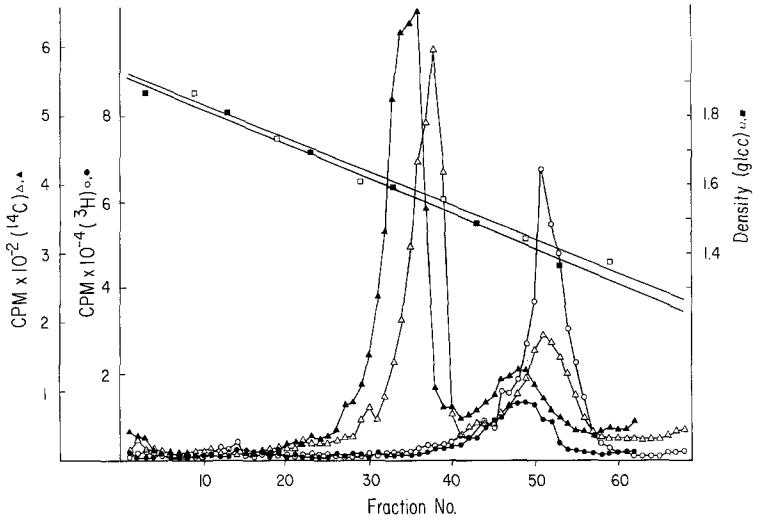

Figure 3: Action of bleomycin on AMV reverse transcriptase product formed in the presence of NaF. Cs_2SO_4 equilibrium density-gradient profile. NaF (0.025 M) was included in the reverse transcriptase reaction mixtures. The symbols are the same as for Fig. 1.

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the ability to degrade the poly(dT) moiety of an RNA-DNA hybrid, while leaving the poly(rA) moiety intact. It should be noted that the poly(rA) portion of the hybrid does not return to the expected density of pure poly(rA) after bleomycin action (Fig. 1). This may be the result of the presence of the $(dT)_{12-18}$ primer (unlabeled) and short regions of the newly synthesized poly(dT) or bound enzyme remaining associated with the poly(rA). The former possibility is not unreasonable, since it is known that bleomycin cannot degrade oligodeoxynucleotides of less than about 3000 daltons molecular weight (5). Further, the presence of the oligo(dT) and/or the enzyme does cause a density shift of the poly(rA) (Fig. 4). The poly(rA) alone has a density of 1.57 g/cc, determined under identical centrifugation conditions (not shown). The nature of the poly(rA) remaining in the hybrid region of the gradient profile after bleomycin treatment is the subject of continuing investigation.

Thus our results provide further evidence for the specificity of action

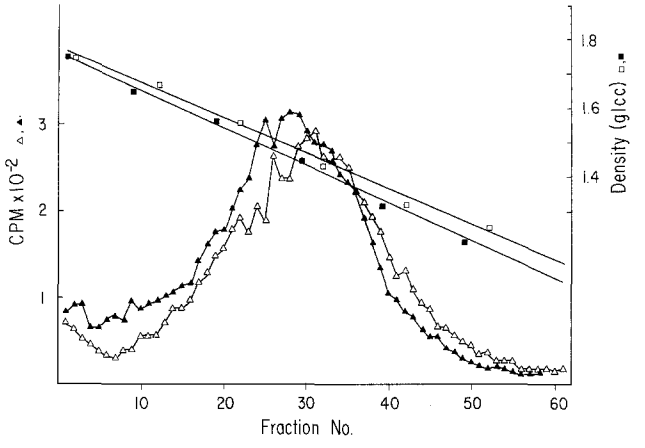

Figure 4: Action of bleomycin on the poly(rA) template. Cs_2SO_4 equilibrium density gradient profile. Sample #1, no bleomycin: △, ^{14}C radioactivity; □, density. Sample #2, + bleomycin: ▲, ^{14}C radioactivity; ■, density.

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of bleomycin toward DNA. As described in an earlier report (6), there are three major structural differences between RNA and DNA. These differences are in base composition (uracil *vs.* thymine), in sugar content (ribose *vs.* deoxyribose), and in secondary structure (differences in helical configuration). We have previously shown that the base composition is not responsible for the specificity, since bleomycin could degrade bacteriophage PBS-1 DNA which contains deoxyuridine in place of thymidine. Additionally it is known that both single- and double-stranded DNAs are degraded (2, 6), whereas neither single- nor double-stranded RNAs are degraded (6). Since the action of the drug in removing the DNA moiety of the RNA-DNA hybrid strongly indicates that the secondary structural configuration is not the factor which confers specificity, then the specificity for bleomycin attack must reside in the sugar moiety.

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