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PREPARATION AND CHEMICAL CHARACTERIZATION OF RADIOIODINATED BLEOMYCIN

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None of the radionuclides with which bleomycin has been labeled have chemical and nuclear properties that are entirely satisfactory for in vivo tumor localization. Bleomycin has been radioiodinated by the iodine monochloride, chloramine-T, and lactoperoxidase methods. Iodine monochloride proved to be the preferred method and conditions were developed whereby 80% of radioiodide was covalently bound to bleomycin. Bleomycin (140 μg) was added to 200 μl of saline/citrate buffer (pH 7.0) followed by radioiodide and iodine monochloride. This reaction mixture was incubated for 1 hr and purified by Sephadex G-10 chromatography. The iodine monochloride reaction product underwent hydrolytic deiodination in vitro at a rate of about 1.2%/day (0.15 M NaCl, 37°C). Bleomycin A and B components were radioiodinated with equal efficiency on a mole fraction basis.

Bleomycin is a chemotherapeutic antibiotic that has been investigated as a potential tumor-localizing radiodiagnostic agent. It exists naturally as a copper (II) ligand (*1*) and it is this chelating ability that has been exploited for all of the isotopic labels reported thus far. Both divalent and trivalent ions that chelate bleomycin have been identified but divalent cations, especially Co^{2+}, form more stable bonds than trivalent cations such as In^{3+} or $SnCl_2$-reduced TcO_4^- (*2*).

Because of the disadvantageous nuclear properties of those radioisotopes that exist chemically as divalent ions (e.g., ^{64}Cu, ^{57}Co, ^{62}Zn) and the instability of higher valence complexes (e.g., ^{111}In, ^{67}Ga, $^{99m}TcO_4^-/SnCl_2$) of bleomycin, we have investigated methods for labeling bleomycin with radioiodine. A covalent iodine–bleomycin bond is likely to be stronger than the metal–bleomycin coordinate bond and, therefore, more resistant to hydrolytic breakdown or competitive displacement. Because ^3H-bleomycin containing no transition metal ions has been shown to clear faster and to be less toxic, yet have equivalent tissue distribution kinetics to Cu^{2+} chelated bleomycin (*3*), a nonchelated bleomycin can be expected to improve the tumor-to-background relationship and reduce the radiation dose. Therefore, ^{123}I-labeled copper-free bleomycin may be the ideal form for this radiodiagnostic agent. We report here a study of bleomycin iodinated by the iodine monochloride, chloramine-T, and lactoperoxidase methods.

MATERIALS AND METHODS

Bleomycin as the sterile copper-free sulfate Blenoxane® (Bristol Laboratories, Syracuse, N.Y.) was dissolved in distilled water to a concentration of 1 mg/ml. Chloramine-T (sodium-N-monochloro-p-toluene sulfonamide) was dissolved in distilled water to a concentration of 1 mg/ml and prepared fresh before each experiment. Lactoperoxidase (E.C. No. 1.11.1.7) was used as supplied by the Sigma Chemical Company, St. Louis, Mo. and was dissolved in 0.05 *M* phosphate buffer to a concentration of 1 mg/ml. Buffers used were 0.067 *M* phosphate, 0.10 *M* borate, and 0.15 *M* saline + 0.02 *M* citrate. All other chemicals were reagent grade and were not purified further.

Radioiodination yields may depend on such reaction conditions as reactant concentration, buffer identity, ionic strength, pH, and duration of reaction. In order to maximize yields, the following standard procedures were adopted. Some procedural varia-

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tions and their effects will be described in the RESULTS section.

Iodine monochloride (*4*) labeling was accomplished by adding 140 μg (0.1 μmoles of bleomycin) to 200 μl of saline/citrate buffer. The radioiodine was added in a volume of 5–500 μl after which 30 μl of 0.0033 *M* iodine monochloride (0.1 μmoles), prepared according to the method of McFarlane (*4*), was added. The reaction mixture was shaken for 5 min and allowed to react for at least 1 hr before preparative separation. Aliquots of the reaction mixture were sampled every 5 min for 2 hr and electrophoresis performed to determine the effective reaction rate.

Chloramine-T labeling (*5*) was accomplished by adding 100 μg of bleomycin to 500 μl of phosphate buffer (pH 7.5), followed by the radioiodine and 25–100 μg of chloramine-T. The reaction was quenched after 5 min by adding 0.25 ml of sodium metabisulfite (1 mg/ml).

Lactoperoxidase labeling (*6,7*) was accomplished by adding 500 μg of bleomycin to 500 μl of phosphate buffer (pH 7.5); radioiodine was added followed by 10 μg of lactoperoxidase and finally 10 μl of 1 m*M* hydrogen peroxide. The mixture was incubated at 37°C for 40 min and quenched by the addition of sufficient cysteine to decompose the hydrogen peroxide.

The analytic separation of I-bleomycin, iodate, and iodide was by electrophoresis (340 V, 6 mA 10 min) on cellulose acetate strips (Sepraphore III, Gelman Instruments, Ann Arbor, Mich.) in sodium barbital buffer (0.05 *M*, pH 8.6). Under these conditions, I-bleomycin stays at the origin and iodate and iodide migrate 2.8 ± 0.8 and 5.8 ± 1.5 cm, respectively. This separation was also used to evaluate the radiochemical purity of *I-bleomycin following a preparative separation. All yields were based on the percent of radioiodine bound to bleomycin as determined by electrophoresis.

The preparative separation of I-bleomycin from I^- and IO_3^- was achieved by Sephadex chromatography. Typical retention volumes are given in Table 1. A Sephadex G-10-120 column was preferred for preparative separations because a shorter column could be used and A and B components of bleomycin are not fractionated. A Sephadex G-25-40 column was used to determine the relative labeling efficiency of the A and B components of bleomycin. Fractions were collected and the specific activity of each determined from spectroscopic (optical density at 280 nm) and radioactivity measurements and recorded in the arbitrary units of counts per minute per optical density. The yields from Sephadex were 95% or more of the electrophoresis yields. Dowex 1X-8 anion-exchange resin is effective in separating I-bleomycin from I^- but was not used when IO_3^- was present.

TABLE 1. ELUTION VOLUMES FOR SEPHADEX COLUMNS: 50 ML BED VOLUME ELUTED WITH 0.15 *M* SALINE

Elution volume	Sephadex G-25-40	Sephadex G-10-120
Void volume	18	30
I-bleomycin A	36	31
I-bleomycin B	43	31
IO_3^-	64	39
I^-	50	92

TABLE 2. BLEOMYCIN IODINATION YIELDS

Reaction	Buffer (pH)	Experiments (No.)	Yield
Iodine monochloride	Saline citrate (6.5)	3	60
	Phosphate (6.5)	3	65
	Saline citrate (7.0)	20	80
	Phosphate (7.0)	3	80
	Saline citrate (7.5)	3	75
	Phosphate (7.5)	3	75
Chloramine-T	Phosphate (7.5)	3	80
Lactoperoxidase	Phosphate (7.5)	4	10–22

The stability of the I-bleomycin purified by Sephadex chromatography has been measured by incubating samples in 0.15 *M* sodium chloride solution at 37°C. Polystyrene test tubes were used, an aliquot was removed at daily intervals for a period of 1 week, and electrophoresis performed to determine the rate at which iodide was liberated.

RESULTS

Labeling chemistry. Labeling yields of bleomycin radioiodinated by the iodine monochloride, chloramine-T, and lactoperoxidase methods are given in Table 2. Both iodine monochloride and chloramine-T methods gave yields of 80% or more based on radioactivity measurements and depending on reaction conditions. Lactoperoxidase yields ranged from 10 to 22% and, therefore, this method of labeling was not pursued.

For the iodine monochloride method, labeling yields were highest between buffers of pH 6.5–7.5, and both phosphate and citrate buffers gave equivalent yields. Labeling efficiencies were unchanged when reaction volumes were varied between 0.5–4 ml. The yield of I-bleomycin progressively increased for 2 hr when iodine monochloride was used but was 60% and 75% of the maximum value at 5 and

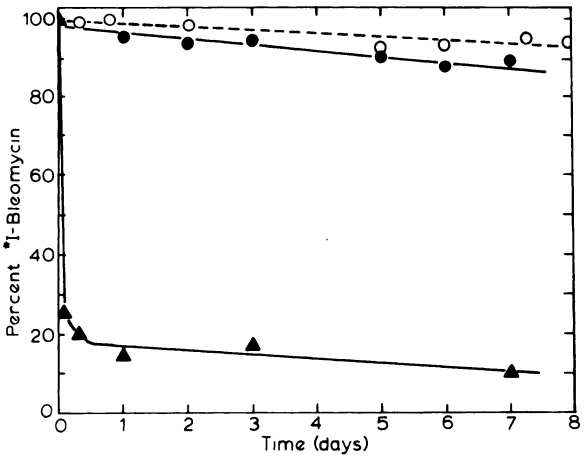

FIG. 1. In vitro hydrolysis of *I-bleomycin. Timing was begun immediately following preparation and purification (Sephadex G-10), and percent *I-bleomycin was determined by electrophoresis. Hydrolysis conditions: 0.15 *M* saline, 37°C. Labeling by iodine monochloride (● and ○ represent two different experiments using the same methods) and chloramine-T (▲). Regression lines - -○- -, Y = 99.7 — 0.84 T, r = 0.903 and —●—, Y = 97.8 — 1.48 T, r = 0.924.

15 min, respectively. The minimum incubation time used for preparative labeling was 1 hr. Optimum labeling efficiency and specific activity of greater than 100 mCi/mg were obtained with 0.14 mg (0.1 μmoles) of bleomycin to which was added 10 mCi ^{125}I-NaI and 30 μl of iodine monochloride.

The chloramine-T reaction yield did not increase with extended incubation time; nevertheless, chloramine-T labeling resulted in a higher specific activity product than iodine monochloride. Due to product instability (see next section), the effects of different reaction conditions on labeling efficiency were not studied further.

In three different experiments, Sephadex G-25 chromatography of *I-bleomycin prepared by iodine monochloride showed equal specific activity for both A and B components. In a typical experiment, the product yields were 18,756 cpm and 8,348 cpm and 0.036 o.d. and 0.016 o.d. for the A and B components, respectively. This corresponds to specific activities (radioactivity/mass $\simeq$ cpm/o.d. of 5.21 $\times$ 10^5 and 5.22 $\times$ 10^5 for the A and B components. The relative concentration of A and B was 69% and 31% of the total bleomycin, respectively, and was constant for the bleomycin which we used.

Stability. The stability of I-bleomycin labeled by the iodine monochloride and chloramine-T methods is shown in Fig. 1. Iodine monochloride-labeled bleomycin undergoes hydrolytic deiodination at a rate of 1.16 $\pm$ 0.32%/day. This is in contrast to the chloramine-T reaction product where 80% is hydrolyzed in the first 8 hr followed by about 1%/day deiodination for the remaining 20% of the product.

DISCUSSION

None of the radionuclides with which bleomycin has been labeled have chemical and nuclear properties which are entirely satisfactory for in vivo tumor localization. Covalent labeling of bleomycin with ^{123}I might provide a tracer bleomycin with a nearly ideal combination of nuclear, chemical, and biologic characteristics for scintigraphic imaging of patients with neoplastic disease.

Iodination of bleomycin by the chloramine-T method was first described by Renault, et al (*8*) but they abandoned it because the product deiodinated in 0.1 *M* phosphate buffer at a rate of 5%/day. We found the chloramine-T reaction product to be even more labile. In the experiment shown in Fig. 1, 85% of the I-bleomycin bonds was hydrolyzed in the first day. This is in contrast to the iodine monochloride reaction product where 1.2% was hydrolyzed each day. These observations suggest that iodine monochloride and chloramine-T cause radioiodine to be bound to bleomycin in different ways.

Lactoperoxidase is useful for radioiodination of large protein molecules containing tyrosine residues but is less effective for histidyl labeling (*6,7*). Bleomycin contains one β-hydroxyhistidine but no tyrosine residues whereas lactoperoxidase contains several tyrosine residues so that at least a 50-fold excess of bleomycin over enzyme must be used in the labeling procedure to minimize self-iodination of the lactoperoxidase. A minimum of 10 μg of enzyme is needed to catalyze the iodination reaction so that 500 μg of substrate bleomycin is required for each iodination. In contrast, the iodine monochloride procedure uses 0.14 mg bleomycin and, therefore, provides a higher specific activity product. From these considerations, we chose to concentrate on iodine monochloride as the agent for bleomycin iodination.

The iodine monochloride labeling yield reached a maximum of 80 $\pm$ 5% at pH 7.0–7.5 for the buffers studied. Whereas the iodine monochloride reaction with tyrosine-containing polypeptides is complete in a few minutes (*4*), the yield for bleomycin continued to increase during the first 2 hr of labeling. The modified histidine residue is the probable site of bleomycin iodination; therefore, the observed rate difference is consistent with the results of other workers who have shown that histidyl residues are less reactive toward electrophilic iodine than are tyrosyl residues (*9*).

The fact that neither the A nor B components of bleomycin are labeled preferentially leads us to believe that the terminal amine residues are not the site of iodination and further, that they do not influence the iodination reaction. The difference in these

two families of bleomycin fractions is that bleomycin A contains sulfur in the terminal amine residues but bleomycin B contains only amines (*10*).

Bleomycin radioiodinated by the iodine monochloride method is stable in vitro and, within the range of selectivity of the chromatography, bleomycin and *I-bleomycin were not distinguishable. The chromatographic methods separate bleomycin based on molecular size as well as on ionic charge properties, so that any lytic or oxidative side reactions caused by iodine monochloride were probably not significant. From our chemical studies, it could, therefore, be anticipated that *I-bleomycin will behave in vivo like bleomycin. Studies in animals, however, will provide a more critical biologic test of this new radiodiagnostic agent. These studies are presently being performed and, although of a preliminary nature, appear promising.

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