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A Radioimmunoassay for Bleomycin

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A radioimmunoassay for bleomycin, suitable for monitoring serum and urine drug levels, has been developed. Antisera were obtained from rabbits immunized with a conjugate of bleomycin and human serum albumin. Bleomycin labeled with ^{57}Co was used as the tracer. Antibody-bound and free bleomycin were separated by precipitation with polyethylene glycol. The assay could determine bleomycin levels down to 0.025 μg/ml. The assay was precise, with a coefficient of variation of 2.8%. Other drugs likely to be administered in combination with bleomycin did not interfere with the radioimmunoassay.

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Bleomycin is a complex polypeptide antitumor antibiotic (*1*) with proven usefulness against a variety of squamous-cell carcinomas, lymphomas, and testicular carcinomas (*2*). While it is relatively nontoxic to bone marrow, its pulmonary toxicity is dose-limiting (*3*). Less frequently, cutaneous toxicity, acute febrile reactions, or hypotension limit its use (*2*).

Existing kinetic and distribution data on bleomycin are based on microbiologic assays (*4–7*) or on assays following direct injection of radioactively labeled bleomycin (*8*). The microbiologic assay is lengthy, with an 18-hr incubation, and is subject to interference from antibiotics. Direct administration of a radioactively labeled drug is not a suitable method for monitoring drug levels.

A rapid assay for serum levels of bleomycin is needed for evaluating various therapeutic regimens, particularly in light of the toxic effects of the drug. A rapid sensitive radioimmunoassay for bleomycin has been reported recently (*9*). That assay utilizes an ^{125}I-labeled bleomycin, two short incubations, and a separation procedure using dextran-coated charcoal. The radioimmunoassay described in this report offers further advantages: a ^{57}Co-labeled bleomycin tracer and separation of bound and free hapten with polyethylene glycol.

MATERIALS AND METHODS

Bleomycin A_2 was coupled to human serum albumin with 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (*10*). Fifty milligrams of human serum albumin (200 μl of 25% salt-poor HSA) was diluted in 20 ml of deionized water. Five milligrams of 2-ethyl-3(3-dimethylaminopropyl)carbodiimide was dissolved in the albumin solution. Twelve milligrams of bleomycin A_2 was added and the solution was stirred for 10 min. Then, another 5 mg of carbodiimide was added and the solution was stirred at room temperature for 24 hr. The bleomycin–albumin conjugate was separated from the reaction mixture in membrane cones.* The conjugate was centrifuged and washed three times with deionized water. Fifty-four milligrams of lyophilized conjugate was obtained.

Four randomly bred New Zealand white rabbits were immunized with 100 μg of the bleomycin–albumin antigen in complete Freund's adjuvant by the multiple intradermal injection method of Vaitukaitis (*11*). Three months after injection, serum was obtained from each rabbit for evaluation of bleomycin antibody.

Cobalt-57 was used to label the bleomycin for the radioimmunoassay. Either bleomycin A_2 or a commercial mixture of bleomycins with bleomycin A_2 as the major fraction† was found to work equally well as the tracer. One hundred microliters of a

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bleomycin solution (250 μg/ml) was added to 500 μCi of ^{57}Co and vortexed. Then 1 ml of 0.5 *M* phosphate buffer, pH 7.4, was immediately added and the resulting solution was diluted to 10 ml with 10 m*M* phosphate buffer, pH 7.4, 0.15 *M* NaCl (PBS). Ninety-five percent of the radioactivity was precipitable with excess antiserum.

To minimize pipetting, the antiserum and ^{57}Co-bleomycin were premixed before the assay. A working solution was made by diluting 100 μl of the ^{57}Co-bleomycin solution to 50 ml with PBS, 1% bovine serum albumin (BSA). The antiserum was diluted 1:600 in PBS, 1% BSA. The antiserum and ^{57}Co-bleomycin solutions were mixed in a 2:1 ratio in sufficient volume to give 300 μl per tube. Standards were prepared from a stock solution of the bleomycin mixture, 100 μg/ml, in PBS, 1% BSA. The stock solution was diluted in human serum that had been treated with charcoal to remove small molecules (*12*). Twenty-five microliters of standards or patient samples was added to 12 × 75-mm culture tubes. Three hundred microliters of the antiserum and ^{57}Co-bleomycin mixture (0.5 ng/20,000 cpm) was added and the tubes vortexed. The tubes were incubated for 60 min at 37°C. One milliliter of 20% (w/v) polyethylene glycol solution‡ was added and thoroughly vortexed. The tubes were centrifuged for 20 min at 2,000 g. The supernatant was aspirated and the precipitates counted for 1 min. Patient values were read from a standard curve, a semilog plot of B/B_0 versus the bleomycin concentration. Patient samples were assayed for endogenous bleomycin antibodies by omitting the rabbit antisera from the assay and substituting a corresponding volume of PBS, 1% BSA.

The radioimmunoassay was used to determine the serum drug levels after bleomycin injection. Bleomycin levels in urine were assayed by the same method, with the addition of 25 μl of bleomycin-free serum after the polyethylene glycol to provide bulk for more effective separation. The bleomycin levels determined by radioimmunoassay were also verified by microbiologic assay.

The ability of other antitumor or antibiotic drugs to displace antibody-bound ^{57}Co-bleomycin was tested by incubating 25 μl of the drug solution with 25 μl of serum and 300 μl of the antibody–^{57}Co-bleomycin solution in the assay procedure. The following agents were tested at concentrations up to 1 mg/ml: actinomycin-D, adriamycin, BCNU, bisulfan, carbenicillin, cyclophosphamide, daunomycin, 5-fluorouracil, gentamicin, keflin, methotrexate, mitomycin, penicillin-G, prednisone, procarbazine, streptomycin, streptozotocin, sulfadiazine, tetracycline, tobramycin, and vincristine.

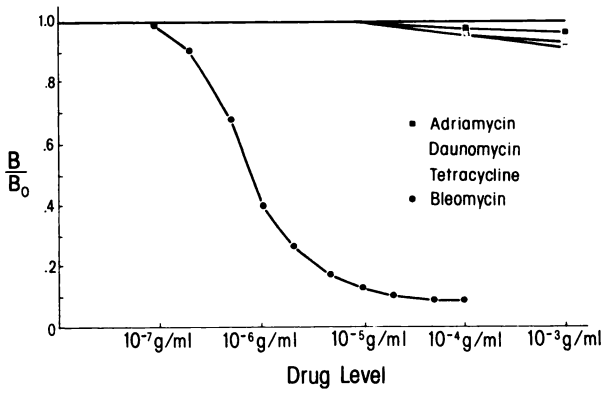

FIG. 1. Binding of ^{57}Co-bleomycin to antibody in presence of added bleomycin (●, standard curve). Adriamycin, daunomycin, and tetracycline displace some radioactivity at high concentrations. Data for other drugs and metal ions tested lie on line $B/B_0 = 1.0$.

Possible interference from metal ions was investigated in the same manner. The concentrations used were either 10 times the normal or 10 times the toxic levels (*13,14*). The metals and concentrations tested were: Ca^{2+} at 110 mg/100 ml; Cd^{2+} at 10 μg/100 ml; Co^{2+} at 0.2 μg/100 ml; Cu^{2+} at 150 μg/100 ml; Fe^{3+} at 1.75 mg/100 ml; Hg^{2+} at 50 μg/100 ml; Mg^{2+} at 30 mg/100 ml; Mn^{2+} at 25 μg/100 ml; Pb^{2+} at 100 μg/100 ml; and Zn^{2+} at 10 mg/100 ml.

RESULTS

Two of the four rabbits immunized produced antibody suitable for use in an assay. The antiserum with the highest titer was used for all studies.

The standard curve (Fig. 1) was usable from 10 μg/ml down to 0.025 μg/ml. This range is broad enough to allow the determination of serum levels and most urine levels without dilution. Dilution was only required for urine samples obtained shortly after administration.

Figure 1 also shows the ability of other drugs to displace the ^{57}Co-bleomycin from the antisera. Of the drugs tested, only tetracycline, daunomycin, and adriamycin had any effect on the binding of ^{57}Co-bleomycin. Their effect was slight and only occurred at levels above their therapeutic range. None of the metals was able to displace ^{57}Co from the ^{57}Co-bleomycin–antibody complex under the assay conditions.

Figure 2 is a plot of the correlation between the radioimmunoassay and the microbiologic assay. The two assays gave nearly identical results, with a correlation coefficient of 0.96. The correlation equation had an intercept very close to zero and the slope was not significantly different from unity.

The assay was reproducible. Ten repetitions of the same patient sample gave an average value of 0.140 ± 0.004 μg/ml for a coefficient of variation

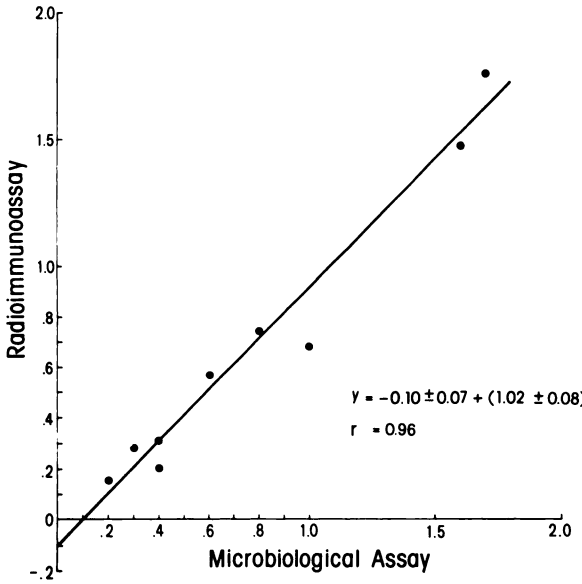

FIG. 2. Correlation of bleomycin levels determined by radioimmunoassay and microbiologic assay.

of 2.8%. Serial dilutions of serum samples with charcoal-treated serum gave bleomycin levels essentially identical to that expected for each dilution.

Bleomycin serum levels peaked and dropped rapidly after intravenous injection. Intramuscular injection resulted in lower peak values but serum levels were maintained for a longer time. These observations are in agreement with data reported for the microbiologic assay.

No endogenous bleomycin antibodies were detected under the conditions of this assay. No serum showed any specific binding of the ^{57}Co-bleomycin tracer, not even the serum from two patients who had exhibited a febrile reaction to bleomycin.

DISCUSSION

The radioimmunoassay for bleomycin is simple and more sensitive than the microbiologic assay. It is specific and not subject to interference from antitumor and antibiotic drugs likely to be administered in combination with bleomycin. While tetracycline, daunomycin, and adriamycin did show a slight effect (Fig. 1), this interference only occurred above the therapeutic levels and should not interfere with the assay in a clinical setting. Metal ions, at concentrations 10 times the normal or toxic levels (*13,14*), did not displace the ^{57}Co from the ^{57}Co-bleomycin–antibody complex under the assay conditions; thus, metal ions are not a likely source of interference.

The radioimmunoassay described in this report offers some advantages over a radioimmunoassay using ^{125}I-labeled bleomycin. The major advantage is the ^{57}Co label. Cobalt-57-bleomycin is easy to prepare, particularly as compared with the procedures required for iodinating the bleomycin and for separating the iodinated from the uniodinated bleomycin. The 271-day half-life of ^{57}Co also offers an obvious advantage, eliminating the necessity for monthly iodine labeling. The ^{57}Co is bound tightly by bleomycin: bleomycin will remove ^{57}Co from a ^{57}Co-EDTA complex when the bleomycin and EDTA are present in equimolar concentrations at pH 6.5 (*15*).

Separation with polyethylene glycol also adds several advantages. Polyethylene glycol precipitates the antibody-bound radioactivity but is much less expensive than a second antibody. A polyethylene glycol separation is easier than a charcoal separation because timing is less important. Charcoal will continue to absorb small molecules as long as it is in contact with the assay solution, whereas polyethylene glycol does not appreciably affect the antibody–hapten equilibrium (*16*). The nonspecific precipitation of the ^{57}Co-bleomycin is low (less than 2%).

Premixing the labeled bleomycin and the bleomycin antisera reduced the number of additions to each assay tube, thereby reducing the "hands on" time and probably increasing the reproducibility. The standards were prepared in serum in order to provide bulk for the polyethylene glycol precipitation of the antibody-bound bleomycin. The charcoal-treated serum used was on hand for T_3 and T_4 assays; any bleomycin-free serum should work as well.

The radioimmunoassay correlated well with the microbiologic assay (Fig. 2). However, several patients' samples were not included in the calculation. With these samples the microbiologic assay had given erroneously high results, up to 50 times the levels indicated by the radioimmunoassay and higher than levels possible with the doses of bleomycin administered. A review of the patients' charts showed that they were receiving other antibiotics. The microbiologic assay, lacking the specificity of the radioimmunoassay, had measured the combined levels of bleomycin and the other antibiotics.

A small percentage of patients have been reported to have an anaphylactoid response to bleomycin (*2*). If this response is mediated by a specific antibody, the antibody might be assayed in the same radioimmunoassay system. Presumably patients with endogenous bleomycin antibody would be poor candidates for bleomycin therapy. Endogenous specific binding of ^{57}Co-bleomycin was not detected by the conditions of this assay, even in patients who had a febrile reaction to low doses of bleomycin.

The microbiologic assay lacks the sensitivity required to determine bleomycin levels directly for

more than several hours after administration. The microbiologic assay has been used to measure rates of bleomycin inactivation by various tissues in order to infer relative bleomycin levels. No clear correlation between in vitro inactivation by neoplastic tissue and in vivo therapeutic effect has been found (*6*). Indeed, the material active in the microbiologic assay may not be the agent with the antitumor activity. Broughton and Strong (*9*) raised the possibility that the radioimmunoassay and the microbiologic assay may measure different parts of the bleomycin molecule. Radioimmunoassay, with its increased sensitivity, may permit direct determination of tissue and serum bleomycin levels that will correlate with antitumor activity.

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FOOTNOTES

* Amicon Centriflo (Lexington, Mass.).

† Blenoxane (Bristol Laboratories, Syracuse, N.Y.).

‡ Carbowax 6000, Union Carbide, Rye, N.Y.

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