RADIOIMMUNOASSAY OF BLEOMYCINS

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(Received for publication April 11, 1985)

Rabbit antisera highly specific to the bleomycinic acid moiety of bleomycins were obtained by immunizing with a conjugate of copper-complex of bleomycin A_5 and bovine serum albumin. These antisera not only reacted with bleomycin A_5 but also with other bleomycins such as bleomycin A_2, bleomycin B_2 and peplomycin. The antisera showed little cross-reactivity with deamido-, depyruvamido- and decarbamoyl-bleomycins. Thus, these antisera were found to be highly specific for the intact bleomycinic acid moiety. One of the antisera was successfully applied to radioimmunoassay of bleomycin and peplomycin in mouse and human sera. The detection limit was 1 ng/ml. This radioimmunoassay is expected to be widely used for the determination of active bleomycins in biological and clinical samples.

Bleomycins (BLM) are a group of antineoplastic antibiotics isolated from *Streptomyces verticillus*[1], and peplomycin (PEP) is a biosynthetic BLM having stronger antineoplastic activity[2]. BLM and PEP have been used in the treatment of several types of malignancies. Various assay methods of BLMs have been developed[3~13], but they are not satisfactory for advanced studies. For instance, HPLC methods were not sufficient in sensitivity without using radio-labeled BLMs[3,4]. Microbial assays were more sensitive but can not be applied to samples containing other antimicrobial substances. Immunoassays were most sensitive and specific among the methods thus far developed[7~13]. However, all the antisera so far reported recognize the terminal amine moiety of BLMs rather than the bleomycinic acid moiety, the active site essential for the antineoplastic activity[8,11~13]. Therefore, those sera were not suitable for the assay of active BLMs. Recently, we were successful in obtaining rabbit antisera which recognized the bleomycinic acid moiety. These antisera were applied to the radioimmunoassay of BLMs. The results are presented in this paper.

Materials and Methods

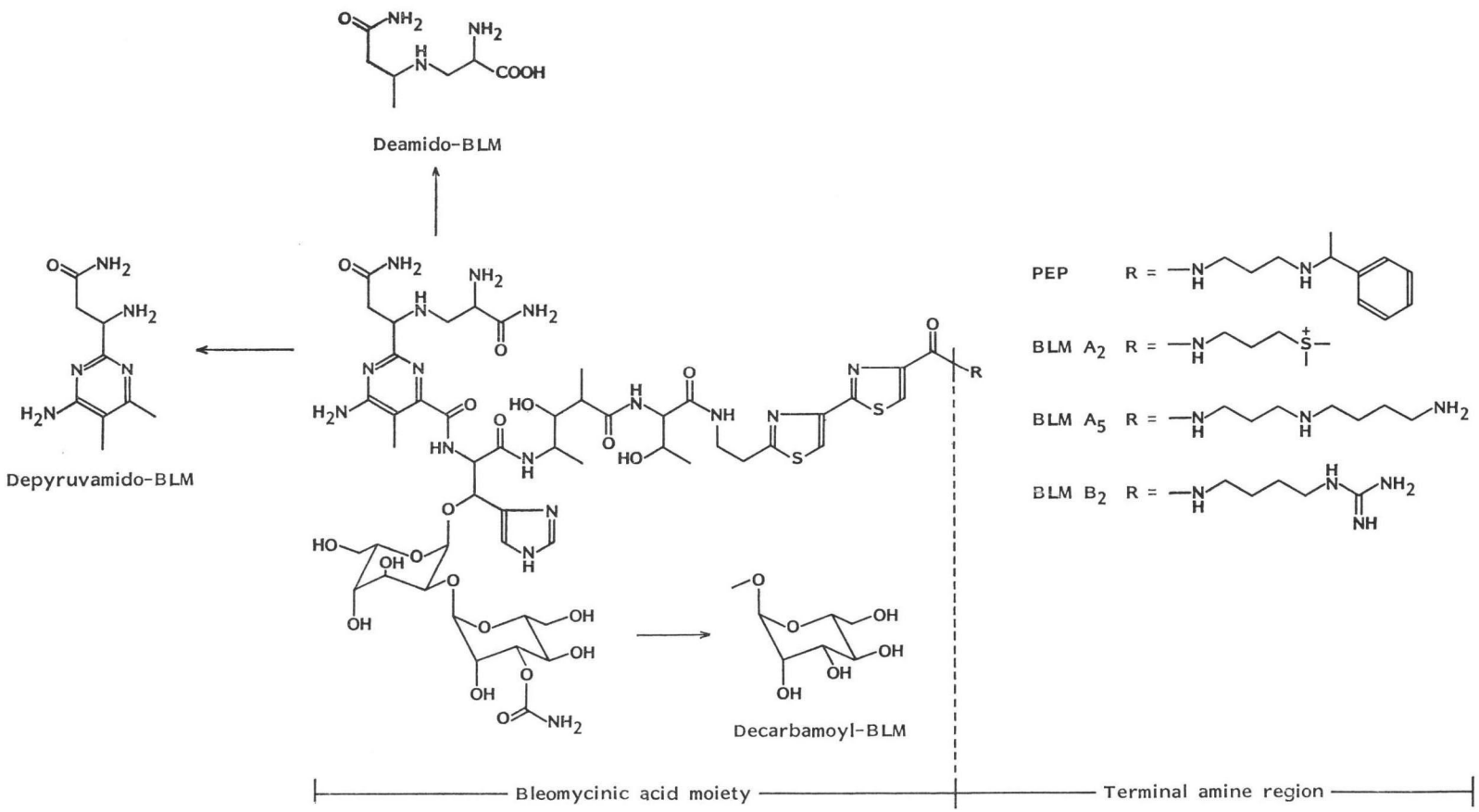
Antineoplastic and Antimicrobial Agents

BLM A_2, A_5 and B_2 and PEP were prepared in Nippon Kayaku Co., Ltd. (Tokyo). Deamido-, depyruvamido- and decarbamoyl-PEPs were prepared according to TAKAHASHI *et al.*[14]. Fig. 1 shows the chemical structure of these seven compounds. [^{3}H]BLM A_2 was prepared from demethyl BLM A_2[15] (specific activity: 5.1 mCi/mol) and [^{3}H]PEP was prepared according to TAKAYAMA *et al.*[3] (specific activity: 0.68 mCi/mol). Other antineoplastic and antimicrobial agents were commercial products for clinical use.

BLM A_5-Bovine Serum Albumin Conjugate

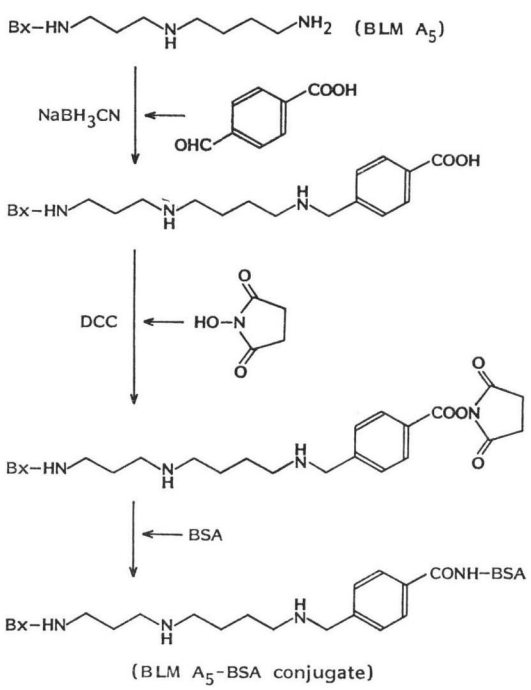
Fig. 2 shows the preparation scheme of a conjugate of BLM A_5 and bovine serum albumin (BSA, Sigma Chemicals Co., Missouri, USA). Blue-colored copper-complex of BLM A_5 (172 mg) and 19.7 mg of terephthalaldehydic acid (Nakarai Chemicals, Ltd., Kyoto) were dissolved in 12 ml of MeOH.

Fig. 1. The structures of BLMs and their derivatives.



To this solution 4.5 mg of sodium cyanoborohydride (Nakarai) was added and the mixture was stirred for 22 hours at room temp. The reaction mixture was adjusted to pH 1 with hydrochloric acid to terminate the reaction, neutralized with sodium hydroxide and evaporated to dryness. The residual materials were dissolved in water and applied to a CM-Sephadex C-25 column (200 ml). The adsorbed materials were eluted by application of a linear gradient formed with each of 500 ml of 0.05 M and 1 M sodium chloride. The blue-colored fraction eluted by around 0.3 M sodium chloride was collected, desalted with an Amberlite XAD-2 column and lyophilized to give a blue powder of *p*-carboxybenzyl BLM A_5-Cu(II). The yield was 127 mg. This powder (50 mg) and 60 mg of *N*-hydroxysuccinimide (Peptide Institute, Osaka) were dissolved in 2 ml of dimethylformamide. The solution was added with 56 mg of *N,N'*-dicyclohexylcarbodiimide (Peptide Institute) and stirred for 20 hours at room temp. The reaction product was collected by precipitation by addition of 20 ml of acetone, washed with acetone, dried, dissolved in water, filtered and lyophilized. The product, *p*-(succinimidooxycarbonyl)benzyl BLM A_5-Cu(II), was a blue powder and the yield was 56 mg. This powder (30 mg) was added to a BSA solution previously prepared by dissolving 40 mg of BSA in 2 ml of 50 mM sodium phosphate buffer, pH 7.5. The mixture was stirred overnight at room temp and subjected to purification by Sephadex G-50 column chromatography. The pale blue reaction product, BLM A_5-BSA conjugate, appeared in the void volume fraction. This was dialyzed against water and lyophilized to give 20 mg of the conjugate. The molar ratio of BLM to BSA in the conjugate was about 7 as calculated from the UV absorbancy.

Fig. 2. Preparation of BLM A_5-BSA conjugate.
Bx; Copper-complex of bleomycinic acid moiety, DCC; *N,N'*-dicyclohexylcarbodiimide.



Anti-BLM A_5 Sera

Five New Zealand white rabbits (1.8~2.2 kg, Tokyo Laboratory Animals Co., Tokyo) were intradermally immunized with the BLM A_5-BSA conjugate. The conjugate dissolved in saline at 1 mg/ml was mixed with the same volume of complete Freund's adjuvant and used for immunization. Immunization was repeated a further three times at one-month intervals. Two weeks after the final immunization all the rabbits were sacrificed and the sera were taken. The sera were incubated at 56°C for 30 minutes to inactivate complement and stored at −80°C until use.

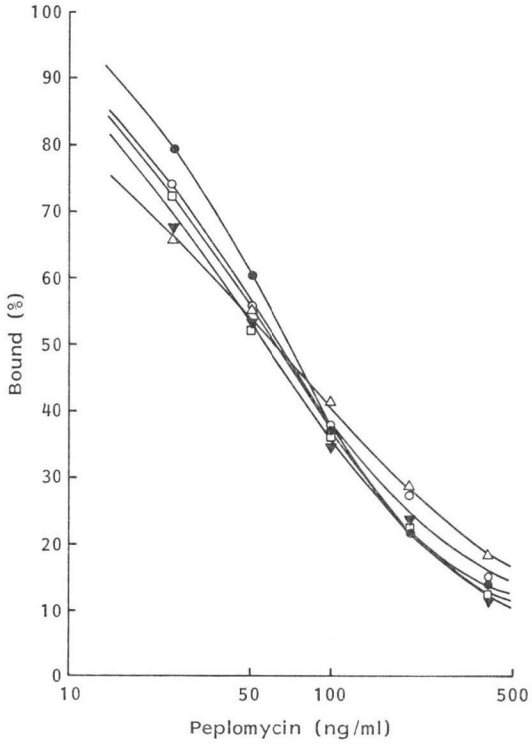
Radioimmunoassay

Anti-BLM serum was diluted with 20 mM phosphate buffer, pH 7.5, containing gelatin 0.2%, NaCl 2.0% and NaN_3 0.2%. The dilution rate was determined depending on the specific radioactivity of the tritiated BLMs used for the assay. For example, when [^{3}H]PEP (0.68 mCi/mol) was used, the dilution rate was 2,000. The diluted antiserum (0.1 ml) was mixed with 0.2 ml of a test sample or a standard BLM-Cu(II) complex solution and 0.1 ml of saline containing [^{3}H]PEP or [^{3}H]BLM A_2 (30,000 dpm). The mixture was incubated first at 37°C for 15 minutes and then in ice water for 15 minutes. The incubated mixture was mixed with 0.1 ml of 0.5% bovine γ-globulin and 0.5 ml of 25% polyethylene glycol 6000 (Wako Pure Chemicals Co., Tokyo). After about 15 minutes, the mixture was centrifuged at 1,800×*g* for 15 minutes and the supernate was removed by aspiration. The precipitate was washed into a scintillation vial with about 1 ml of distilled water. The radioactivity was

Table 1. Reactivity of rabbit anti-BLM A_5-BSA sera to BLM's and BLM analogs as determined using [^{3}H]PEP.

Analog	Reactivity (%)				
	Antiserum No.				
	1	2	3	4	5
PEP	100	100	100	100	100
BLM A_2	74.5	148.4	86.7	70.9	79.5
BLM A_5	111.3	78.5	157.8	120.3	106.7
BLM B_2	98.5	162.8	98.5	118.2	85.0
Bleomycinic acid	98.9	48.4	96.3	84.5	86.1
Deamido-PEP	16.8	5.8	5.9	3.6	2.6
Depyruvamido-PEP	2.2	2.8	3.8	1.5	0.8
Decarbamoyl-PEP	1.4	1.3	0.6	1.8	0.3

Fig. 3. Radioimmunoassay of PEP using [^{3}H]PEP (0.68 mCi/mol).
Antiserum: 1 (○), 2 (△), 3 (▼), 4 (□), 5 (●).



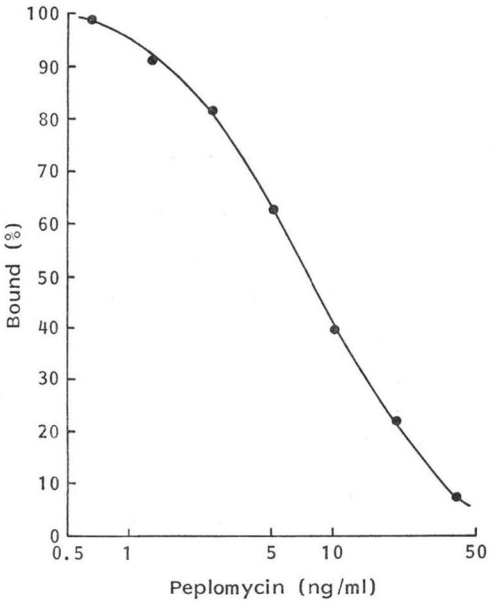
determined after addition of Scintillant (ACS II, Amersham).

Results and Discussion

BLM A_5 has two primary amino groups, one in the bleomycinic acid moiety and another in the terminal amine region (Fig. 1). The amino group at the bleomycinic acid moiety is protected by coordination with cupric ion[16]. Therefore, the amino group in the terminal amine region is involved in the conjugation with BSA. Thus, the antisera raised toward this conjugate were expected to recognize the bleomycinic acid moiety. This expectation was confirmed by the following immunochemical examination.

When PEP was radioimmunoassayed with the five antisera using [^{3}H]PEP, all the antisera gave excellent PEP dose-responses and the dose-response curves were similar to one another (Fig. 3). In addition, these antisera similarly reacted with BLM A_2, A_5 and B_2 (Table 1). The antisera also cross-reacted with bleomycinic acid. However, the cross-reactivity of BLM derivatives modified in the bleomycinic acid moiety (deamido-, depyruvamido- and decarbamoyl-BLMs) was much lower. Therefore, it was found that these antisera specifically recognize the intact bleomycinic acid moiety. All of the previously reported antisera had much less specificity toward the bleomycinic acid moiety[8,11~13]. This should be due to the fact that the previous immunogen conjugates were prepared by coupling with the amino group of the bleomycinic acid moiety of BLM[7~13]. Deamido-, depyruvamido- and decarbamoyl-BLMs are biologically inactive. Therefore, this radioimmunoassay is expected to be useful for determination of bioactive BLMs.

Fig. 4. Radioimmunoassay of PEP using [^{3}H]BLM A_2 (5.1 mCi/mol) and antiserum No. 5.



The cross-reactivities of the antisera to other antineoplastic agents such as cisplatin, doxorubicin, mitomycin C, methotrexate and vinblastin were less than 0.01% of that of PEP. The cross-reactivities to β-lactam antibiotics such as piperacillin, ticarcillin and cefoperazone were less than 0.0001%.

The sensitivity of the radioimmunoassay using the [^{3}H]PEP was 6.5 ng/ml (Fig. 3). When the [^{3}H]BLM A_2, which has 7.5 times higher specific radioactivity than the [^{3}H]PEP, was used, the detection limit reached 1 ng/ml (Fig. 4). The radioimmunoassay using the [^{3}H]BLM A_2 was tested in the serum of mice injected with PEP intravenously. Microbial assay was also done with the same samples for comparison. The results are shown in Fig. 5. The detection limit of the radioimmunoassay was about 1 ng/ml, while that of the microbial assay was about 50 ng/ml. The correlation co-efficient between both data was 0.988 as calculated after logarithmic conversion of the data. When this assay was applied to the determination of PEP in a mixture of PEP and human serum, the dose response was low. However, when this mixture was previously deproteinized by addition of 2 volumes of ethanol, an excellent dose response curve was obtained (data not shown). Therefore, it is expected that active BLM and PEP in biological and clinical samples can be determined with sensitivity and precision by the radioimmunoassay presented in this paper.

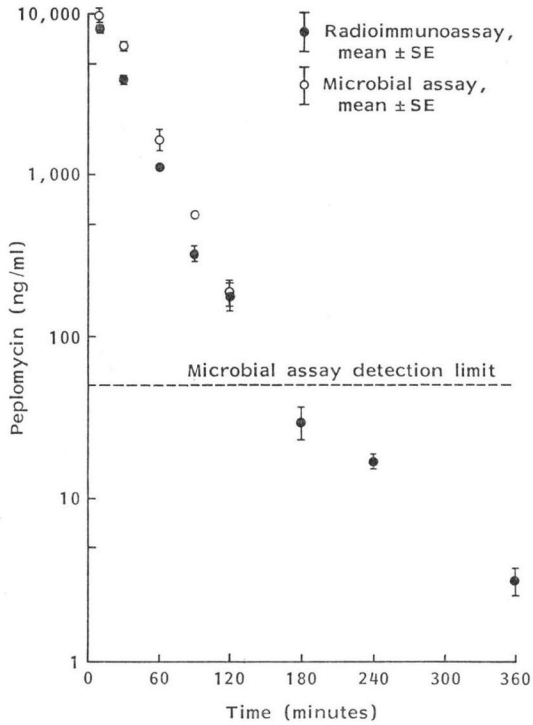
Fig. 5. PEP level in mouse serum.

PEP (5 mg/kg) was injected into 5 week-old ICR mice (Shizuoka Laboratory Animal Center, Hamamatsu) *via* the tail vein and the mice were sacrificed at indicated times to take the sera. A group consisted of 3 mice.

These sera were then subjected to PEP determination by both radioimmunoassay and microbial assay. The microbial assay was done as described below.

A serum sample or standard PEP was soaked to a paper disk (6 mmϕ) as much as possible and put on a nutrient agar plate. The agar plate contained 5 g of peptone, 3 g meat extract, 15 g agar in a total volume of 1,000 ml and spores of a BLM-sensitive mutant of *Bacillus subtilis* PIC219.

The plate was incubated first in a refrigerator for 2 hours and then at 30°C for 18 hours. PEP concentration was estimated from the diameter of the growth-inhibited zone.



Acknowledgments

We wish to thank Mr. F. ABE and Mr. K. SHIBUYA for immunization of rabbits and Mr. H. EKIMOTO for technical supports in pharmacokinetic experiments. We also thank Dr. T. NAKATANI for suggestions for preparation of this article.

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