IMMUNOLOGICAL CROSS-REACTIVITIES OF VARIOUS ANTHRACYCLINE ANTIBIOTICS AGAINST ACLACINOMYCIN A ANTISERA

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Aclacinomycin A (ACM) antisera were obtained from the rabbits immunized with 4'''-deoxy-4'''-(R)-amino-ACM- or *N,N*-didemethyl-ACM-bovine serum albumin conjugate, and their immunoreactivities were tested with ACM-related anthracyclines.

It was found that the binding ability with the ACM antisera was markedly decreased by the following structural changes in ACM: *N,N*-didemethylation of the rhodosamine moiety; 6-*O*- or 4-*O*-methylation; removal of the methoxycarbonyl group at C-10; hydroxylation at C-1, C-2 or C-11. It was less affected by some alterations in a side chain at C-9 or by deglycosidation of the terminal mono or disaccharide. The binding of the aglycone (aklavinone) was very weak.

Keywords—aclacinomycin A; radioimmunoassay; aclacinomycin A antisera; anthracycline; rabbits

Aclacinomycin A (ACM) is a new anthracycline antibiotic with therapeutic usefulness¹⁾ and is produced by *Streptomyces galilaeus* MA144-M1.²⁾ This drug is an aklavinone trisaccharide (AKN-RhoN-deFuc-Cin abbreviated as cinerulosyl-2-deoxyfucosyl-rhodosaminylaklavinone)^{2a)} and is metabolized in mammals or tissue to several glycosidic metabolites, such as MA144 M1, N1, S1 or T1 (aklavin), and some non-glycosidic metabolites such as 7-deoxyaklavinone, MA144 E1 or aklavinone.³⁾ For a quick and sensitive assay for ACM in a biological fluid, we have developed a radioimmunoassay (RIA) of ACM.⁴⁾

This paper deals with the production of ACM antisera in rabbits immunized with four kinds of ACM-BSA conjugates, the specificities of two potent ACM antisera thus obtained, and the immunological cross reactivities with other anthracycline antibiotics having the same trisaccharide as ACM.

MATERIALS AND METHODS

Chemicals and Reagents — *N*-Succinimidyl-

[2,3-³H]propionate (50 Ci/mmol) was purchased from The Radiochemical Center, Amersham. Aclacinomycin A (ACM), *N,N*-didemethyl ACM (MA144 K1), MA144 M1, MA144 S1, MA144 T1 (aklavin) and MA144 L1^{2b)} or MA144 U5,⁵⁾ 13-methylaclacinomycin A,⁶⁾ cinerubin A,⁷⁾ baumycins,⁸⁾ 4-hydroxybaumycins,⁹⁾ feudomycin A¹⁰⁾ and roseorubicin A¹¹⁾ were obtained from the cultured broths of *Streptomyces galilaeus* MA144-M1, *Streptomyces coeruleorubidus* ME130-A4, *Actinomadura* sp. D326, *Actinomyces roseoviolaceus* A529 and their mutant strains. Various anthracyclines having a cinerulosyl-2-deoxyfucosyl-rhodosaminyl residue as the glycosidic moiety were prepared by either microbial or chemical modification.¹²⁾ Auramycin A and sulfurmycin A were kindly supplied by Dr. Fujiwara, Nippon Roche. 4'''-deoxy-4'''-(R)-aminoaclacinomycin A (4'''-(R)-NH₂ ACM) was prepared from ACM by chemical modification.¹³⁾ Adriamycin and daunomycin were kindly provided by Dr. Arcamone, Farmitalia C. Erba, Milan and carminomycin I from

Bristol Laboratories, Syracuse.

Preparation of Hapten-BSA Conjugate — To a solution containing 8 mg of 4'''-(R)-NH₂ACM or MA144 K1 and 20 mg of bovine serum albumin (BSA, Sigma Co.) in 1 ml of distilled water was added 20 mg of CDI (1-ethyl-3-(dimethylamino-propyl)carbodiimide·HCl, Protein Research Foundation, Osaka). After the solution was stirred overnight at room temperature, the hapten-BSA conjugate was separated from the unreacted ACM hapten (4'''-(R)-NH₂ACM or MA144 K1) and CDI by Sephadex G-75 (ϕ1.8×40 cm) column chromatography using buffered saline (0.15 M NaCl, 0.005 M phosphate, pH 7.0).

Glutaraldehyde coupling for the conjugation was carried out as follows. To a solution of 20 mg of BSA and 8 mg of 4'''-NH₂ACM or MA144 K1 in 2 ml of 0.1 M phosphate buffer (pH 7.0) was added in dropwise 1 ml of 0.25% aqueous glutaraldehyde. After 2 h, 1 ml of 1 M aqueous lysine (pH 7.0) was added to consume the unreacted glutaraldehyde. Thereafter the reaction mixture was chromatographed as described above to separate the hapten-BSA conjugate from low-molecular weight species.

Preparation of ACM Antisera — The hapten-BSA conjugate (10 mg) was dissolved in 5 ml of saline, emulsified with the same amount of complete Freund's adjuvant and injected to a male rabbit (New Zealand White) at the foot pads (0.5 ml per foot pad) once a week for two months. The antiserum obtained one week after the final booster injection was kept at -20°C until use.

Preparation of Radiolabeled Hapten — 4'''-(R)-*N*-[2,3-³H]propionyl ACM: 4'''-(R)-NH₂ACM (20 nmol) dissolved in 20 μl of tetrahydrofuran and 80 μl of 0.05 M phosphate buffer (pH 7.5) were added into the small test tube containing 20 nmol of *N*-succinimidyl[2,3-³H]propionate. After the reaction mixture was stirred at 30°C for 60 min, the product, 4'''-(R)-*N*-[2,3-³H]propionyl ACM, was extracted with toluene, transferred to 0.05 M acetate buffer (pH 3.5) to remove the unreacted *N*-succinimidyl[2,3-³H]propionate, and re-extracted with toluene after adjusting pH to 7.0 with 0.1 M

phosphate buffer.

N-[2,3-³H]propionyl ACM: *N*-[2,3-³H]propionylation of MA144 K1 (20 nmol) with *N*-succinimidyl[2,3-³H]propionate in tetrahydrofuran and phosphate buffer (pH 7.0) was performed as described above. The toluene extract was concentrated to dryness under reduced pressure and the residue was chromatographed on a column (ϕ1.2×40 cm) of Sephadex LH-20 with methanol as eluent. The eluate in fraction tube No. 29 and 30 (1.4 ml/tube) containing *N*-[2,3-³H]propionyl ACM were pooled, concentrated under reduced pressure and stocked at -20°C until use.

RIA Procedure — Radioimmunoassay (RIA) was performed by the competitive binding procedure in a homologous system. One hundred μl of physiological buffer (0.01 M phosphate, 0.01 M EDTA, 0.15 M NaCl and 0.1% gelatin, pH 7.4), 100 μl of test anthracycline serially diluted with saline containing 0.1% gelatin, 10 μl (about 10000 dpm) of the ³H-propionyl ACM solution, and 100 μl of ACM antiserum at 100-fold dilution were mixed and incubated at 4°C for 2 h. After the incubation, 200 μl of dextran-coated charcoal (0.05% dextran T-70, Pharmacia Fine Chemical Co. and 0.5% activated charcoal, E. Merck Co., in the physiological buffer) was added to remove free ³H-labeled and unlabeled anthracyclines. Therefore, the mixture was centrifuged at 2500 rpm for 10 min. The radioactivity of ³H-ACM hapten bound with antibodies in 400 μl of the supernatant was measured with BRAY's cocktail using an Aloka LSC-653 scintillation spectrometer. All analyses were carried out in duplicate, and the values were corrected for the nonspecific binding of ³H-ACM hapten to normal rabbit serum.

RESULTS AND DISCUSSION

³H-ACM Haptens, ACM Antigens and ACM Antisera

For labeling of ACM with tritium and for conjugation of ACM with BSA, 4'''-deoxo-4'''-(R)-aminoACM (4'''-(R)-NH₂ACM) and *N,N*-didemethylACM (MA144 K1) which have a pri-

mary amino group at C-4''' and C-3' positions were used, respectively. Highly specific ^3H -labeled ACM haptens were prepared by propionylation of these compounds with *N*-succinimidyl[2,3- ^3H]propionate. The specific radioactivities of 4'''-*N*-[2,3- ^3H]propionyl and *N*-[2,3- ^3H]propionyl ACM haptens thus obtained were about 9 and 15 Ci/mmol, respectively.

Four ACM-BSA conjugates used as ACM antigens were also prepared from 4'''-(R)-NH₂ACM

or MA144 K1 by coupling with BSA using either CDI or glutaraldehyde. The binding ratio of ACM hapten to BSA molecule was determined by absorbance at 435 nm and ranged from 1 to 18 mol per BSA molecule (Table I). When rabbits were immunized with these antigens (1 mg) every week for two months, the titer of ACM antibodies in each immunized rabbit was different ranging from 8 to 250 as shown in Table I. 4'''(R)-NH₂ACM-BSA conjugate (CDI coupling) and MA144 K1-BSA conjugate (glutaraldehyde

TABLE I. ACM Antisera produced in Rabbits immunized respectively with Four Kinds of ACM-Hapten-BSA Conjugates

Antigen	Coupling ^{a)} method	ACM/BSA molar ratio	Antiserum ^{b)} activity
4'''-(R)-NH ₂ ACM-BSA	C	2.9	250
4'''-(R)-NH ₂ ACM-BSA	G	1.0	64
MA144 K1-BSA	C	18.3	8
MA144 K1-BSA	G	5.3	250

a) C: CDI coupling; G: glutaraldehyde coupling.

b) Dilution of antiserum to the activity that give a 50% displacement of ^3H -ACM hapten in RIA described in Text.

MA144 K1-BSA

4'''-(R)-NH₂ACM-BSA

hyde coupling), which contained 3 to 6 mol of ACM per BSA molecule, yielded high titers of ACM antisera which were designated antiserum A and B, respectively. The immunization of MA144 K1-BSA conjugate (CDI coupling) which contained about 18 mol of ACM per BSA molecule, did not give a more potent ACM antiserum. Calibration curves for ACM using antisera A and B are shown in Fig. 1.

Specificities of ACM Antisera

The immunological stereospecificities of antisera A and B were tested with ACM, its glycosidic and non-glycosidic metabolites in mammals or MA144 K1. The results are shown in Table II, in which the cross reactivity was expressed as a ratio of test compound/ACM in the concentrations giving a 50% displacement of ^3H -ACM in RIA for ACM. Both antisera reacted with the biologically active metabolites (MA144 M1, S1 or T1 (aklavin)) as strongly as ACM, but showed little or no binding with the biologically inactive non-glycosidic metabolites such as aklavinone. These findings imply that metabolic removal of the terminal mono or disaccharide give only a slightly reduced effect on the immunoreactivity

with both antisera. Some difference in the immunoreactivity between antisera A and B was observed when MA144 K1 and aklavinone were tested. Antiserum A reacted with MA144 K1 at the binding rate as only about one-tenth as ACM, while antiserum B reacted with it as strongly as ACM. Furthermore, in contrast with antiserum A, antiserum B exhibited still a slight but significant binding activity against aklavinone. These findings show that antiserum A has a more restricted immunospecificity against ACM metabolites than antiserum B. In other words, antiserum A had a more specific affinity for *N,N*-dimethylaminosugar (rhodosamine) moiety of trisaccharide in ACM molecule but antiserum B did for the aglycone moiety rather than the sugar moiety. The above immunoreactive properties against ACM metabolites allow their use for RIA of ACM or its glycosidic metabolites in a biological fluid. In fact, we had developed a method for RIA of ACM using antiserum A, in which a linear calibration curve was obtained at a range of 1 to 10 pmol per assay.⁴⁾

Cross-Reactivities of Other ACM-Related Anthracyclines

TABLE II. Cross-reactivities of Aclacinomycin Analogs in Aclacinomycin A-RIA

	Compound	R	Cross-reactivity (%) ^{a)}	
			Antiserum A	Antiserum B
ACM	-RhoN-deFuc-Cin	100	100
	MA144 M1	-RhoN-deFuc-Ami	96.0	100
	MA144 S1	-RhoN-deFuc	96.7	95.0
	MA144 T1	-RhoN	78.4	100
	MA144 K1	-DauN-deFuc-Cin	13.8	94.3
	Aklavinone	-H	0.05	7.5

<p>RhoN: L-rhodosamine Dau: L-daunosamine deFuc: 2-deoxy-L-fucose Cin: L-cinerulose A Ami: L-amicetose</p>				

a) Cross-reactivity (%) is calculated as the concentration of ACM required for 50% inhibition / the concentration of tested compounds required for 50% inhibition $\times 100$

FIG. 1. Calibration curves with ACM
—●— using antiserum A and —○— antiserum B.

The immunoreactivities of antisera A and B were further examined against related anthracyclines in order to obtain some information about the similarity or dissimilarity in the molecular conformation between ACM and other anthracyclines, as well as to manifest their immunospecificities in more details (Table III).

6-*O*-Methyl, 4-*O*-methyl and 10-demethoxycarbonyl ACMs chemically derived from ACM,^{12 c, d)} being less active against leukemic L1210 cells than ACM,¹⁴⁾ showed a considerably reduced binding with the ACM antisera. Their cross-reactivities were less than 20% with antiserum A, and their cross-reactivities were around 1% with antiserum B although 6-*O*-methyl ACM was an exception in this case. The serological binding of 6-*O*-methyl ACM with antiserum B occurred similarly as with ACM. 13-Methyl ACM, sulfurmycin A and auramycin A, in which the ethyl group at C-9 position of ACM was replaced by isopropyl, acetonyl and methyl groups, respectively, reacted with antiserum A at a cross-reactivity over 70% and with antiserum B at that

TABLE III. Cross-reactivities of Aclacinomycin Analogs in Aclacinomycin A-RIA

Compound	R1	R2	R3	R4	R5	R6	R7	Cross-reactivity (%)	
								Anti-serum A	Anti-serum B
ACM	H	H	OH	OH	H	COOCH ₃	CH ₂ CH ₃	100	100
13-Methyl-ACM	H	H	OH	OH	H	COOCH ₃	CH(CH ₃) ₂	90.5	52.9
Sulfurmycin A	H	H	OH	OH	H	COOCH ₃	CH ₂ COCH ₃	84.4	53.3
Auramycin A	H	H	OH	OH	H	COOCH ₃	CH ₃	63.9	60.0
Cinerubin A	OH	H	OH	OH	H	COOCH ₃	CH ₂ CH ₃	10.7	26.5
ε-RMN-A	H	H	OH	OH	OH	COOCH ₃	CH ₂ CH ₃	15.3	26.5
ε-IsoRMN-A	OH	H	OH	OH	OH	COOCH ₃	CH ₂ CH ₃	6.6	10.0
β-RMN-A	H	H	OH	OH	OH	OH	CH ₂ CH ₃	2.6	0.5
γ-RMN-A	H	H	OH	OH	OH	RN-dF-C (C7:H)	CH ₂ CH ₃	<0.01	0.2
2-Hydroxy-ACM	H	OH	OH	OH	H	COOCH ₃	CH ₂ CH ₃	0.1	0.3
4- <i>O</i> -Methyl-ACM	H	H	OCH ₃	OH	H	COOCH ₃	CH ₂ CH ₃	0.1	1.4
6- <i>O</i> -Methyl-ACM	H	H	OH	OCH ₃	H	COOCH ₃	CH ₂ CH ₃	19.3	100
10-Demethoxycarbonyl-ACM	H	H	OH	OH	H	H	CH ₂ CH ₃	10.4	0.5
Trisarubicinol	H	H	OH	OH	OH	H	CH(OH)CH ₃	1.9	0.5
Daunomycinone-A	H	H	OCH ₃	OH	OH	H	COCH ₃	<0.01	0.05

ACM: aclacinomycin A, RMN: rhodomycinone.

TABLE IV. Cross-reactivities of Adriamycin Analogs in Aclacinomycin A-RIA

Compound	R1	R2	Cross-reactivity(%)	
			Antiserum A	Antiserum B
Carminomycin	OH	COCH ₃	< 0.01	0.6
Daunomycin	OCH ₃	COCH ₃	< 0.01	< 0.01
Adriamycin	OCH ₃	COCH ₂ OH	< 0.01	< 0.01
Feudomycin A	OCH ₃	CH ₂ CH ₃	< 0.01	0.4

of more than 50%. Thus, the alteration of the side chain at C-9 affected the immunoreactivity with the ACM antisera only to a small extent.

Cinerubin A (1-hydroxy ACM) and ϵ -rhodomycinone-A (11-hydroxy ACM) showed 10 to 16% of the cross-reactivity in the immunoreactivity with antiserum A and both 26.5% in that with antiserum B, suggesting that the hydroxyl group at either C-1 or C-11 interferes the serological binding activity of both ACM antisera to a great extent. The presence of two hydroxyl groups at C-1 and C-11, as seen with ϵ -isorhodomycinone A (1,11-dihydroxy ACM), thus caused an additive-down effect on the immunoreactivity. 2-Hydroxy ACM, which had been found to have an excellent antitumor effect on leukemic L1210-bearing mice,¹⁵⁾ scarcely reacted with both antisera (less than 1% cross-reactivity). Therefore, the hydroxyl group at C-2 produces a more heavily steric hindrance for receptor sites of ACM antibodies than those at C-1 and C-11.

β -Rhodomycinone A (10-demethoxycarbonyl-10, 11-hydroxy ACM), γ -rhodomycinone A binding to the trisaccharide moiety at C-10, trisarubicinol (dihydrocarminomycinone-A) and daunomycinone-A did not exhibit the serological affinity for both antisera (Table III), although they have the same trisaccharide as in ACM. Adriamycin, carminomycin, daunomycin and feudomycin A were also insensitive as shown in Table IV. It seems likely that these results are reasonable since they are significantly different

either in aglycone moiety or glycosidic moiety or both from ACM.

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