SCREENING AND SOME PROPERTIES OF NEW MACROMOLECULAR PEPTIDE ANTIBIOTICS

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In searching for macromolecular antitumor antibiotics of microbial origin, 2,875 kinds of Actinomycetes culture fluids were applied to a newly developed test system which consisted of antimicrobial assay using a macromolecule permeable mutant, DNA damage assay and mutagenicity test. As a result, 78 macromolecular antibiotics were found. Among them, 15 antibiotics precipitable with ammonium sulfate were macromolecular peptide antibiotics (protein antibiotics), of which molecular weight ranged from 10,000 to 14,000.

Macromolecular peptide antibiotics AN-1, -5 and -15, termed type I antibiotics, showed stronger growth inhibitory effect on the *uvrA* and *recA* mutants, as compared to the effect on their parent, MP2. They also had mutagenic activity. AN-7, -9, -16, -18, -20, -22, -23, -25, and -26, termed type II, exhibited an increased inhibitory activity to a *recA* mutant but did not to an *uvrA* mutant. They all showed mutagenicity. AN-3, -11 and -13, type III antibiotics, gave similar influence on the DNA repair mutants, and on their parent, MP2. They had no mutagenic activity. Except for AN-11 and -13 of type III antibiotics, all antibiotics were inhibitory to the cell growth of a cancer cell, L1210.

Our previous paper[1] described a novel microbial system that was applicable to the screening of macromolecular antitumor antibiotics of microbial origin. The system consisted of 1) the system for isolation of macromolecular antibiotics based on macromolecule permeable property of a mutant, MP2[2,3], derived from *Escherichia coli* K-12, 2) the system to detect inhibitor of DNA synthesis employing DNA repair mutants derived from MP2 and 3) the system to detect mutagenic activity of newly isolated antibiotics using a valine sensitive strain of MP2, whose conversion rate to valine resistance was dependent on mutagenic activity.

In this paper, the newly established test system was applied for screening of macromolecular peptide antibiotics producing Actinomycetes. As a result, 15 kinds of macromolecular peptide antibiotics were newly discovered. Among them, 13 samples showed inhibitory effect on the *in vitro* cell growth of a cancer cell, L1210.

Materials and Methods

Microorganisms

Escherichia coli K-12 W3876 and its macromolecule permeable mutant, MP2, were employed for the detection of macromolecular antibiotics. REC9, UV28, UR3 and L7 were derived from MP2 and described in the preceding paper[1].

A number of Actinomycetes were newly isolated from various soil samples.

Cultivation of Actinomycetes

Actinomycetes were cultivated at 29°C in shake flasks. The culture media used were described in

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Table 1. Production media for macromolecular peptide antibiotics.

(1) GW	Glucose	3.0%	(4) GSS	Starch	2.5%
	Polypepton	0.5		Glucose	1.0
	Meat extract	0.5		Polypepton	0.5
	NaCl	0.5		Meat extract	0.5
	$CaCO_3$	0.3		K_2HPO_4	0.1
	pH	7.0		NaCl	0.3
				$CuSO_4 \cdot 5H_2O$	100 μg/ml
(2) GWD	0.5% dried yeast was added to the GW			$FeSO_4 \cdot 7H_2O$	1 μg/ml
				$MnCl_2 \cdot 4H_2O$	1 μg/ml
(3) GM	20 μg/ml $MnCl_2 \cdot 4H_2O$ was added to the GWD and $CaCO_3$ was replaced by 0.2% $CaCl_2 \cdot 2H_2O$			pH	7.0
			(5) GSCZ	500 μg/ml $ZnSO_4 \cdot 5H_2O$ was added to the GSS	

Table 1. The supernatant of each culture broth after centrifugation was subjected to the screening test.

Determination of Antibacterial Activity

Quantitative determination of antibacterial activity was done by the disk assay using UR3[1]. The diameter of inhibition zone was proportional to the logarithm of antibacterial activity. One unit of antibacterial activity was defined as the antibiotic concentration to give 10 mm (diameter) of inhibition zone.

Cell Culture of L1210

In order to examine antitumor activity of newly isolated antibiotics, their inhibitory effect on the growth of cancer cells was examined. A cancer cell, L1210 was cultured in Eagle minimum essential medium supplemented with 10% calf serum, 5 μg/ml of cefazolin and 100 μg/ml of streptomycin at 37°C in a 7% CO_2 incubater.

Analysis

Molecular weight of macromolecular peptide antibiotics was determined by chromatography on a column (1.5×30 cm) of Bio-Gel P30 using 0.02 M phosphate buffer containing 0.1 M NaCl (pH 7.0) as an eluting buffer (flow rate was 0.05 ml/minute). Blue dextran 2,000, myoglobin (MW 17,600), cytochrome C (MW 12,500) and bacitracin (MW 1,400) were used as standard.

BIURET, ninhydrin and anthrone reactions were done by the method of WESTLEY and LAMBETH[4], BLACKBURN[5] and SPIRO[6], respectively.

Results

Screening of Macromolecular Antibiotics

Culture fluids of various Actinomycetes were subjected to the macromolecular antibiotic detecting system in which macromolecule permeable mutant MP2 was employed. As reported in the preceding paper, among 2,875 samples tested 78 samples showed strong growth inhibitory effect on MP2[1]. Therefore, the molecular weight of these antibiotics seemed to be more than 1,200. Furthermore, active fractions of 15 out of 78 samples were precipitated from culture fluids by saturating with ammonium sulfate. These 15 samples were chosen as macromolecular peptide antibiotics to be investigated further in detail.

DNA Damage Assay and Mutagenicity Test for 15 Antibiotics

The 15 newly isolated antibiotics were subjected to both DNA damage assay and mutagenicity test, and classified into three groups as shown in Table 2. An *uvrA* mutant, UV28 was more sensitive to DNA binding antibiotics such as actinomycin D, mitomycin C and adriamycin as compared to its parental strain MP2[1]. On the other hand, a *recA* mutant of MP2, REC9 became more sensitive not only to the DNA binding antibiotics but also to the DNA degrading antibiotics such as neocarzinostatin

Table 2. Effect on DNA repair and mutagenesis of various macromolecular peptide antibiotics.

Type	Antibiotics	Antibacterial activity on*			Mutagenic activity**
		UV28 (*uvrA*)	REC9 (*recA*)	UR3 (*uvrA*, *recA*)	
I	AN-1, -5, -15	+	+	+	+
II	AN-7, -9, -16, -18, -20, -22, -23, -25, -26	−	+	+	+
III	AN-3, -11, -13	−	−	−	−

* 100 units/ml of antibiotics solution was used. +: Stronger inhibitory effect on repair mutant. −: No difference in growth inhibition between repair mutant and its parent, MP2.

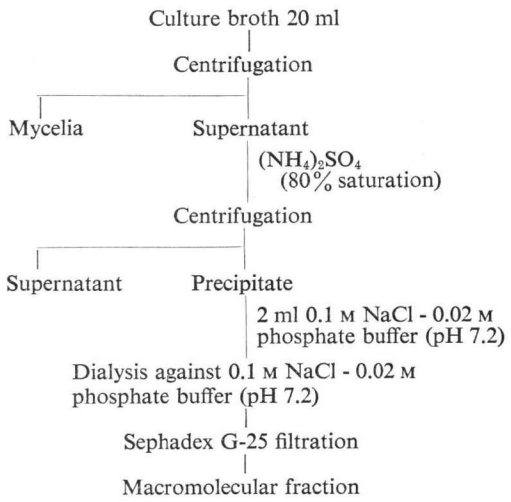
** The mutagenic activity was measured by valine resistance test as described in Materials and Methods. +: Positive, −: negative.

and bleomycin[1]. Type I antibiotics, which included AN-1, -5 and -15, showed slightly increased toxicity to both UV28 and REC9 and also had mutagenic activity. Type II, which included AN-7, -9, -16, -18, -20, -22, -23, -25 and -26, showed increased toxicity to REC9, but did not show it to UV28. Type II also showed mutagenicity. Therefore, properties of type II antibiotics were similar to that of neocarzinostatin. Type III, which included AN-3, -11 and -13, did not exhibit increased toxicity both to UV28 and REC9. Mutagenic activity was not detected in type III antibiotics.

Purification and Some Properties of 15 Antibiotics

The 15 different macromolecular peptide antibiotics were purified according to the procedure shown in Fig. 1. The active fraction was precipitated by adding ammonium sulfate. The precipitate was dissolved in 0.02 M phosphate buffer (pH 7.2) containing 0.1 M NaCl and then dialyzed for 24 hours at 5°C against the same buffer. The nondialyzable protein was applied to Sephadex G-25 gel filtration. More than 20% of the activity was recovered from the culture broth with this purification procedure. Specific activity of each sample ranged between 17 and 820 units/mg protein as shown in Table 3. Some physicochemical properties were presented in Table 4. Molecular weight of 15 antibiotics ranged from 10,000 to 14,000 and UV maxima of the samples existed between 252 and 278 nm. These properties indicated polypeptide nature of the antibiotics. AN-18 and AN-20 gave positive Anthrone reaction and their UV maxima was 252 nm. Therefore, the two antibiotics may contain nucleoside structure in their molecules.

Fig. 1. Isolation procedure of macromolecular peptide antibiotics.



Effect of Newly Isolated Macromolecular Peptide Antibiotics on the Growth of L1210 Cells

Antitumor activity of the 15 newly isolated macromolecular antibiotics was examined by utilizing *in vitro* culture of L1210 cells. As shown in Table 5, all of the antibiotics belonging to the type I and II exhibited antitumor activity. Among them, AN-1, -5 of type I and AN-7, -16, -22, -23 and -25 of

Table 3. Production of macromolecular peptide antibiotics and their antibacterial activities.

Antibiotics	Producing strains	Production media	Antibiotics produced (unit/ml)	Specific antibacterial activity of partially purified fraction (unit/mg protein)
AN- 1	*S. albus* AJ9003	GWD	50	326
AN- 3	AAP-23	GSS	42	80
AN- 5	G-36	GW	50	120
AN- 7	B-19	GM	50	820
AN- 9	B-20	GSS	120	820
AN-11	AAC-4	GSS	42	80
AN-13	AAP-21	GSS	42	80
AN-15	*S. albus* AJ9081	GSS	17	80
AN-16	J-24	GM	8	720
AN-18	No. 454	GSCZ	84	17
AN-20	No. 463	GSCZ	84	17
AN-22	I-101	GM	50	650
AN-23	I-502	GM	50	650
AN-25	K-506	GM	50	720
AN-26	No. 163	GWM	140	34

Table 4. Some characteristics of macromolecular peptide antibiotics.

Antibiotics	Molecular weight $\times 10^4$	Color reaction*			UV_{max}
		Biuret	Ninhydrin	Anthrone	
AN- 1	1.1~1.3	++	+	±	270 nm
AN- 3	1.3~1.4	++	+	−	278
AN- 5	1.2~1.3	++	+	−	270
AN- 7	1.0~1.3	++	+	±	270
AN- 9	1.2~1.3	++	+	±	270
AN-11	1.3~1.4	++	+	−	278
AN-13	1.3~1.4	++	+	−	278
AN-15	1.3~1.4	++	+	−	278
AN-16	1.0~1.3	++	+	−	270
AN-18	1.0~1.3	++	+	+	252
AN-20	1.0~1.3	++	+	+	252
AN-22	1.0~1.3	++	+	−	270
AN-23	1.0~1.3	++	+	−	270
AN-25	1.0~1.3	++	+	−	270
AN-26	1.3~1.4	++	+	−	276

* 1.5 mg/ml of solution was used.

type II showed prominent activity in that the antibiotic concentration giving 50% growth inhibition was as low as 0.25 unit/ml. Two samples, AN-11 and -13 out of three antibiotics of type III had no effect on growth of L1210. However, AN-3 of type III showed marked inhibitory effect on the growth of L1210 cells.

Table 5. Effect of macromolecular peptide antibiotics on growth of mouse leukemia L1210 cells.

Type*	Antibiotic	Growth inhibition of L1210**							
		Dose (unit/ml)							
		10	5	2.5	1	0.5	0.25	0.1	0.05
I	AN-1	+++	+++	+++	++	++	+	+	−
	AN-5	+++	++	++	+	−	−	−	−
	AN-15	+	−	−	−	−	−	−	−
II	AN-7	+++	+++	+++	+++	+++	++	+	−
	AN-9	+	−	−	−	−	−	−	−
	AN-16	+++	+++	+++	+++	+++	++	+	−
	AN-18	++	+	−	−	−	−	−	−
	AN-20	++	+	−	−	−	−	−	−
	AN-22	+++	+++	+++	+++	+++	++	+	−
	AN-23	+++	+++	+++	+++	+++	++	+	−
	AN-25	+++	+++	+++	+++	+++	++	+	−
	AN-26	+++	+	−	−	−	−	−	−
III	AN-3	+++	+++	+++	++	++	−	−	−
	AN-11	−	−	−	−	−	−	−	−
	AN-13	−	−	−	−	−	−	−	−

* See Table 2.

** −, No growth inhibition; +, about 25% inhibition; ++, about 50% inhibition; +++, 100% inhibition.

Discussion

Macromolecular peptide antibiotics such as neocarzinostatin[7~10] and macromomycin[11~14] appeared particularly important and useful as antitumor agents as they are expected to have a high specificity and strong activity against tumor cells as compared to small molecular antibiotics. From this viewpoint, we developed a microbial screening system for a macromolecular antitumor antibiotics as described in our previous paper[1]. We applied this new assay system to many kinds of Actinomycetes culture fluids, and succeeded to obtain 15 macromolecular antibiotics. These antibiotics appeared to be mostly polypeptides of about 10,000 daltons (Table 4). Since they were not extensively purified, some nonproteinaceous characteristics such as absorption maximum other than 280 nm might be merely due to contaminants. Thus, an approximate frequency to find macromolecular peptide antibiotics was one in every 200 strains of Actinomycetes.

There are more than a dozen of proteinaceous antibiotics so far reported, but only a few have been studied carefully[15~26]. On first inspection, most of the known macromolecular antibiotics appeared to be different from ours in regard to their chemical and biological properties.

Table 6. Grouping of newly isolated macromolecular peptide antibiotics.

Interaction with DNA	Mutagenic activity	Macromolecular peptide antibiotics obtained by our screening	Known antitumor antibiotics
Detected with DNA damage assay			
DNA-binding type	Positive	AN-1, -5, -15	Mitomycin C
	Not detected	Not found	Actinomycin D
DNA-degrading type	Positive	AN-7, -9, -16, -18, -20, -22, -23, -25, -26	Neocarzinostatin
	Not detected	Not found	Bleomycin
Not detected with DNA damage assay			
	Not detected	AN-3, -11, -13	

The antibiotics obtained were classified into three groups in relation to their properties of interaction with DNA and mutagenic activities (Table 6). Type I antibiotics had DNA-binding activity and showed mutagenicity. Adriamycin and mitomycin C seemed to belong to this group but such protein-like antibiotics has not probably been reported previously. Type II had characteristics of DNA-degrading type rather than DNA binding. They also showed mutagenicity. Biological activities of this group's antibiotics resembled those of neocarzinostatin. Type III showed neither DNA-binding nor DNA-degrading activity and had no mutagenic activity.

Newly isolated antibiotics of type I and II are expected to have antitumor activity since they inhibited *in vitro* cell growth of L1210. Their antitumor activities are now being tested in an *in vivo* assay. Among type III antibiotics, only AN-3 had inhibitory effect on L1210 cell growth. AN-3 showed neither DNA-binding nor degrading activity. But our further study on AN-3 indicated that it inhibited the incorporation of [^{3}H]thymidine into DNA. Therefore, AN-3 was considered to interact with DNA inhibiting its synthesis in an unknown manner.

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