A NEW TEST SYSTEM FOR SCREENING MACROMOLECULAR ANTITUMOR ANTIBIOTICS AND ITS APPLICATION TO CULTURE FLUIDS OF ACTINOMYCETES

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In searching for macromolecular antitumor antibiotics, a new screening method was developed that consisted of 1) a macromolecular antibiotic detecting system employing macromolecule permeable mutants of *Escherichia coli*, 2) a system to detect DNA-affecting antibiotics using DNA repair mutants, and 3) a mutagenicity detecting system, employing a valine resistance test. This new test system was applied to about 2,900 kinds of culture fluids of Actinomycetes and consequently 15 samples were found which contained macromolecular antibiotics with DNA affecting properties.

Several useful antitumor antibiotics possessing polypeptide structure have been found in culture fluids of Actinomycetes. They include actinomycin D[1], neocarzinostatin[2], bleomycin[3], macromomycin[4] and sporamycin[5]. Among them, neocarzinostatin, bleomycin and actinomycin D are clinically employed in Japan. Common properties of these compounds are: 1) They are large molecules with molecular weight ranging from 1,200 to 13,000 and 2) they interact with DNA causing the inhibition of DNA synthesis[6~9].

Macromolecular peptide antibiotics appear particularly important and useful as antitumor agents because they have higher specificity in action and stronger activity against tumor cells as compared to smaller molecule antibiotics. However, screening of such macromolecular antibiotics seemed to be hampered due to the lack of appropriate assay methods.

Recently, one of us reported on a macromolecule permeable mutant of *E. coli* K-12, MP2, which is sensitive to macromolecules such as neocarzinostatin (a protein antibiotic) and proteinase[10,11]. Therefore, we attempted to employ the mutant for the development of a microbial system that is applicable to the prescreening of macromolecular antitumor antibiotics of microbial origin.

In this paper we describe the microbial test system, which consists of 1) the system for isolation of macromolecular antibiotics based on macromolecule permeable property of strain MP2, 2) the system to detect inhibitors 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 derived from MP2 whose conversion to valine resistance was especially sensitive to mutagenic compounds.

Some results obtained by the application of this system are also given.

Materials and Methods

Strain

Table 1 shows the list of the strains and their genetic markers employed in this work. Construction of MP2 derivatives was done by mating MP2 with suitable Hfr donor strains (Table 1). Among many

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Table 1. Bacterial strains used.

Strain	Mating type	Relevant genotype	Other marker or derivation	Source or reference
W3876	F⁻	wild	*lac*11D3 *mal*15 str-r *lys trp leu* T6ʳ	H. UCHIDA
MP1	F⁻	*mmpA*	*mmpA* derived from W3876 by NG	(10)
MP2	F⁻	*mmpA mmpB*	*mmpB* derived from MP1 by NG	(10)
L7	F⁻	*mmpA mmpB* val^s	leu$^+$ derived from MP2 by mating with 17-9	This paper
UV28	F⁻	*mmpA mmpB uvrA*	*uvrA* and leu$^+$ derived from MP2 by mating with 17-9	〃
REC9		*mmpA mmpB recA*	*recA* and lys$^+$ derived from MP2 by mating with KL-16-99	〃
UR3		*mmpA mmpB uvrA recA*	*recA* and lys$^+$ derived from UV28 by mating with KL-16-99	〃
17-9	HfrC	*uvrA*	*trp his* str-r *uvrA* 54	H. OGAWA
KL16-99	Hfr	*recA*	*recA*1	(12)

Mmp: macromolecule permeable character.
NG: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

recombinant colonies, appropriate recombinants having desired phenotypes were selected, purified and tested for each character. Techniques used in these analyses were similar to those described by MILLER[13].

Media

PM medium contained Polypepton 0.5%, meat extract 0.5% and NaCl 0.3%. Difco antibiotics medium 3 (M3) was used throughout this study, unless otherwise mentioned. M9V contained glucose 0.2%, NH_4Cl 0.1%, Na_2HPO_4 0.6%, KH_2PO_4 0.3%, $MgSO_4 \cdot 7H_2O$ 0.02%, NaCl 0.05%, nutrient broth 0.008%, lysine 50 μg/ml, tryptophan 50 μg/ml, valine 50 μg/ml and agar 1.5%.

Determination of Antibacterial Activity

Test-tube Test: 0.05 ml of an overnight culture of *E. coli* strain (*ca.* 10^3 cells/ml) was inoculated into 2 ml of PM medium containing various concentrations of a drug in a small test tube (120 mm in length, 9 mm in diameter). After incubating at 37°C for 20 hours, an optical density at 660 nm of the culture liquids was measured with a spectrophotometer. The minimal concentration of an antibiotic causing 100% growth inhibition was determined.

Disk Assay: Overnight culture (0.05 ml) of an *E. coli* strain was added into 2 ml of PM medium containing 0.5% agar at 42°C. M3 plate containing 1% agar was overlaid with the PM soft agar. The paper disks of 8 mm diameter soaked with 0.05 ml of various concentrations of drug solution were placed on the above mentioned agar plate. The plate was incubated at 37°C for 20 hours and diameter of the inhibition zone was measured. Minimal inhibitory concentration was defined as the concentration to give a minimal inhibition zone on the plate.

Mutagenic Test

In order to detect mutagenic activity of antibiotics, 0.1 ml of overnight culture (10^8 cells/ml) of a valine sensitive derivative of MP2, strain L7, was spread on M9V plate, on which paper disk containing 0.05 ml of each sample solution was placed. The plate was incubated at 37°C for 4 days. The number of valine resistant colonies that were grown around the paper disk containing an antibiotic were then counted.

His⁻ reversion test was carried out by the method of AMES with and without S9 fraction[14].

Chemicals

Neocarzinostatin and bleomycin were gifts of Kayaku Antibiotics Research Company and Nippon Kayaku Company, respectively. Chemicals were obtained from the following sources: benzyl penicillin, Takeda Chemical Co.; bacitracin, Tokyo Kasei Kogyo Ltd.; actinomycin D, Merck Co.; adriamycin and mitomycin C, Kyowa Hakko Company.

Results

Effect of Molecular Size of Antibiotics on Growth of MP1 and MP2

Table 2 shows minimal inhibitory concentrations of antibiotics having various molecular weights to *E. coli* W3876 and its derivatives. Growth of strains MP1 and MP2 was inhibited by a low concentration of neocarzinostatin, bleomycin, actinomycin D, polymyxin B, erythromycin and rifampicin, although some of the drugs had little effect on the parental strain. The molecular weight of these compounds is larger than 700. However, MP2 was more sensitive than MP1 to neocarzinostatin, bleomycin, actinomycin D, bacitracin and polymyxin B, whose molecular weight is more than 1,200. Therefore, MP1 and MP2 appeared to be useful in searching for various macromolecular antibiotics.

Effect of Antibiotics on DNA Repair Mutants

An *uvrA* mutant of MP2, UV28 became sensitive to antibiotics of the DNA-binding type such as actinomycin D[15], mitomycin C[16] and adriamycin[17]. A *recA* mutant of MP2, REC9 became sensitive to the DNA-degrading antibiotics such as neocarzinostatin[18] and bleomycin[19] in addition to the DNA-binding antibiotics. Based on these results, it was reasoned that DNA-binding and -degrading antibiotics could be separated with the simple assay method employing both UV28 and REC9 mutants.

Table 2. Inhibitory effect of antibiotics against cell growth of *E. coli* W3876, MP1 and MP2.

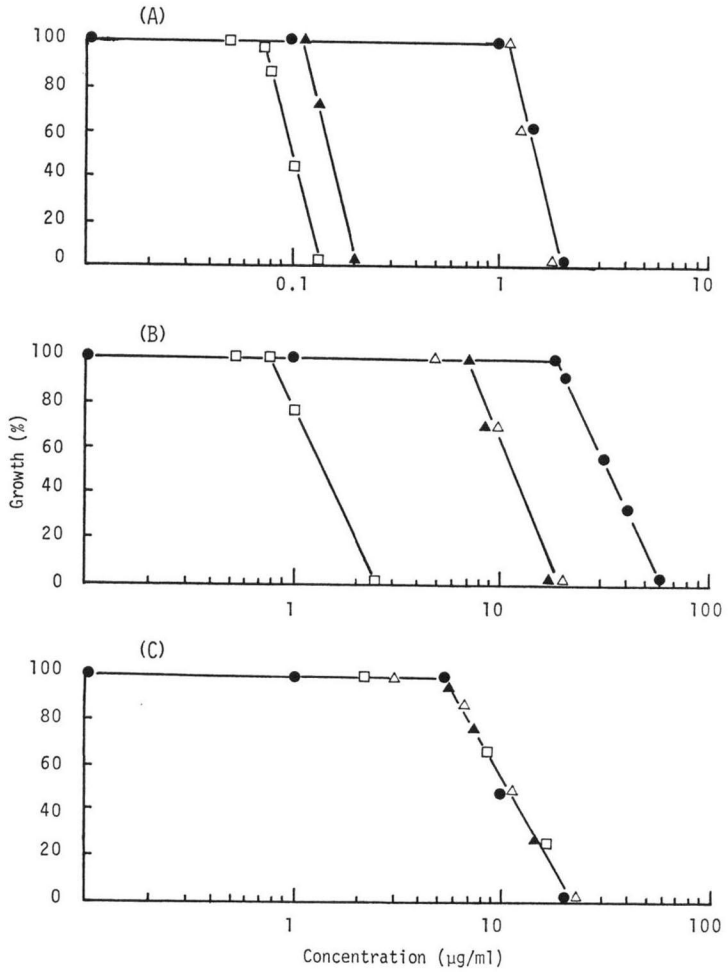
Antibiotics	MW	Inhibition site	MIC (μg/ml)			Relative sensitivity		
			W3876	MP1	MP2	W3876/MP1	W3876/MP2	MP1/MP2
Neocarzinostatin	10,700	DNA	>1,000	10	2	>100	>500	5
Bleomycin	1,300~1,800	DNA	20	1.5	0.3	13	67	5
Actinomycin D	1,255	DNA	>1,000	300	50	>3	>20	6
Mitomycin C	334	DNA	5	3	3	1.7	1.7	1
Adriamycin	550	DNA	>500	>500	>500	1	1	1
Rifampicin	809	RNA	40	1.5	1.5	27	27	1
Bacitracin	1,400	Cell wall	>200	>200	150	1	>1.3	>1.3
Benzylpenicillin	334	Cell wall	400	250	200	1.6	2	1.3
Polymyxin B	1,200	Cell membrane	150	80	20	1.9	7.5	4
Erythromycin	734	Protein	50	1	1	50	50	1
Chloramphenicol	323	Protein	15	13	13	1.2	1.2	1

Minimal inhibitory concentration (MIC) was determined by disk assay, which is described in Materials and Methods. Relative sensitivity were indicated with ratio of MIC of antibiotics against *E. coli* strains.

Table 3. Inhibitory effect of antibiotics against cell growth of *E. coli* DNA repair mutants.

Antibiotics	Interaction with DNA	MIC (μg/ml)				Relative sensitivity		
		MP2	UV28	REC9	UR3	MP2/UV28	MP2/REC9	MP2/UR3
Neocarzinostatin	Yes, degrading	2	2	0.2	0.14	1	10	14
Bleomycin	Yes, degrading	0.3	0.3	0.01	0.01	1	30	30
Actinomycin D	Yes, binding	50	20	20	2.5	2.5	2.5	20
Mitomycin C	Yes, binding	3	1	0.3	0.1	3	10	30
Adriamycin	Yes, binding	>500	50	20	2	>10	>25	>250
Rifampicin	No	1.5	1.5	1.0	1.0	1	1.5	1.5
Bacitracin	No	150	150	150	150	1	1	1
Benzylpenicillin	No	200	200	200	200	1	1	1
Polymyxin B	No	20	20	20	20	1	1	1
Erythromycin	No	1	1	1	1	1	1	1
Chloramphenicol	No	13	13	13	13	1	1	1

Fig. 1. Concentration effect of marcomolecular antibiotics on growth of *E. coli* DNA repair mutants.
The *E. coli* strains used for the test-tube test of macromolecular antibiotics were MP2 (●), UV28 (△), REC9 (▲) and UR3 (□). Macromolecular antibiotics are neocarzinostatin (A), actinomycin D (B) and polymyxin B (C).



An *uvrA* and *recA* double mutant, UR3 seemed particularly important for selecting inhibitors of DNA synthesis, since the degree of the sensitivity in UR3 was most profound to those inhibitors, especially to actinomycin D, adriamycin and mitomycin C. The sensitivity of these three kinds of mutants to antibiotics such as bacitracin, benzylpenicillin, chloramphenicol and rifampicin which are inhibitors of cell wall, cell membrane, protein or RNA synthesis, did not change at all (Table 3). Fig. 1 illustrates a characteristic dose response of the above three kinds of mutants to neocarzinostatin (DNA-degrading), actinomycin D (DNA-binding) and polymyxin B (inhibitor of cell membrane). The kind and the mode of action of newly isolated antibiotics can be assumed by determining the minimal inhibitory concentration of the antibiotics to these mutants.

Mutagenicity Test of Macromolecular Antibiotics

Mutagenic activity of several kinds of antitumor antibiotics was examined with AMES his$^-$ reversion test[14] and our valine resistance test as shown in Table 4. Bleomycin and actinomycin D did not show

Table 4. Effect of macromolecular antibiotics on mutagenesis.

Antibiotics	Mutagenic activity		
	Valine resistance test	His⁻ reversion test	
	L7	TA-100	TA-98
Neocarzinostatin	+++	−	−
Bleomycin	−	−	−
Actinomycin D	−	−	−
Bacitracin	−	−	−
Polymyxin B	−	−	−

Valine resistance test was performed by method which is described in Materials and Methods. His⁻ reversion test employing *Salmonella typhimurium* TA100 and TA98 was described by AMES *et al.*[14]

Concentration of antibiotics: 20 μg/paper disk.

+++: Positive to induce many valine resistance mutant, −: negative.

Addition of S9 fraction gave same results.

any mutagenic activity in either system. However, neocarzinostatin exhibited mutagenic action only in valine resistance system but not in his⁻ reversion test. TATSUMI *et al.* reported very weak mutagenicity of neocarzinostatin in *E. coli* B strain[20]. Therefore, it was shown that by using the new test system macromolecular antibiotics could be differentiated into two types; one with mutagenic activity such as neocarzinostatin and the other without mutagenic activity like bleomycin.

Assay Program for Antitumor Antibiotics

We developed a new assay program for antitumor antibiotics of microbial origin by combining the three test system mentioned above. As illustrated in Table 5, in the first step, antimicrobial compounds with molecular weight more than 700 can be identified by employing MP1, and compounds with mo-

Table 5. Screening program for macromolecular antibiotics with DNA-interacting property.

Step I: Isolation of macromolecular antibiotics			Step II: Isolation of DNA-affecting antibiotics			Step III: Mutagenicity test	Expected properties of antibiotics (Example)
Strains used							
W3876	MP1	MP2	UR3 (*uvrA*, *recA*)	UV28 (*uvrA*)	REC9 (*recA*)	L7	
+	+	+					MW: <700 (Mitomycin C)
−	+	+					MW: 700~1,200 (Erythromycin)
−	−	+	−	−	−		MW: >1,200, No direct interaction with DNA (Bacitracin)
			+	+	+	+	MW: >1,200, DNA-binding
						−	MW: >1,200, DNA-binding (Actinomycin D)
			+	−	+	+	MW: >1,200, DNA-degrading (Neocarzinostatin)
						−	MW: >1,200, DNA-degrading (Bleomycin)

Step I; +: growth inhibition, −: no growth inhibition.

Step II; +: stronger inhibitory effect on repair mutant, −: no difference in growth inhibition between repair mutant and its parent, MP2.

Step III; the mutagenic activity was measured by valine resistance test, +: positive, −: negative.

lecular weight above 1,200 by MP2. In the second step, the substances which interact with DNA could be detected by an *uvrA*, *recA* double mutant. Then the mode of interaction with DNA might be differentiated by using each of *uvrA* and *recA* mutants. Furthermore, mutagenic activity of macromolecular antibiotics may be determined with the valine resistance test.

Screening of Antitumor Antibiotics by New Assay System

Our assay system for selecting new antitumor antibiotics was applied to 2,875 kinds of culture fluids of Actinomycetes. Seventy eight samples whose activity seemed to be associated with macromolecular substances showed antimicrobial activity. These culture fluids were applied to DNA damage assay and only 15 samples appeared to contain substances interacting with DNA.

Among 15 samples, 12 showed mutagenic activity and 3 samples had no mutagenicity.

Some of these macromolecular antibiotics thus found were isolated from culture fluids and purified to some extent. Properties of these antibiotics including antitumor activity have been studied and will be described in the accompanying paper.

Discussion

Antibiotics with macromolecular structure may have both advantages and disadvantages. Physiologically active macromolecules generally show a high specificity in action and a powerful activity as exemplified by enzymes and peptide hormones. Therefore, macromolecular antibiotics were thought to be particularly suited as therapeutic agents against tumor cells whose essential differences from normal cells still remain quite obscure. On the other hand, the possible disadvantages of macromolecular drugs may be their relatively lower permeability into the target cells and the possible antigenicity which may bring about undesirable side effects. However, the lastly mentioned property might be advantageous because certain macromolecules have immunopotentiation effect on the host animal.

Since the exploration of macromolecular antibiotics seemed to be not enough, we attempted to search for new ones by a bacterial test system. To screen macromolecular chemicals, it was necessary to detect biological activity and to discriminate macromolecules from other antibiotics. An unique mutant of *E. coli* K-12, MP2 appeared quite useful for this purpose. The difference among the parental strain W3876, MP1 and MP2 in sensitivity to certain chemicals is simply due to the molecular size of a chemical and not due to the mode of its action, as illustrated in Table 2.

Thus, any substances showing greater growth inhibitory activity on MP2 or MP1 than on their parental strain had molecular weight larger than 700. There has been no exception for this rule. Although some wild-type bacteria have sensitivity to macromolecular antibiotics similar to MP1 or MP2, they can not be utilized for screening big antibiotics because they are sensitive to antibiotics of various sizes.

The system to detect macromolecular inhibitor of DNA synthesis also appeared to be efficient in searching antitumor agent. Since most of the antitumor drugs used up to now are known to have some influence on DNA replication, they are more inhibitory to the growth of DNA repair mutant than to the parental strain[15,21]. However, we did not restrict ourselves to screen DNA-damaging type antibiotics. DNA-reacting antitumor drugs are often mutagenic to various degrees, but non-mutagenic drug is desirable. Therefore, we developed a test system to measure mutagenicity of macromolecular antibiotics, for which other bacterial tests were not applicable due to the permeability barrier.

Macromolecular antibiotics reported previously appeared to be isolated either fortuitously during the ordinary screening program or by the time-consuming methods utilizing mammalian cell culture. The method described here seemed to be entirely novel. Like other microbial screening system, this method has been proved to be very efficient and useful in performing the first step screening. In fact we have already isolated several valuable macromolecular antitumor agents. Properties of these agents will be described in our subsequent papers.

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