A METHOD OF SCREENING FOR ANTIBIOTICS PRODUCING OXYGEN RADICALS

Sir:

Several antitumor agents such as streptonigrin[1], anthracyclines[2,3] *etc.* produce oxygen radicals, for instances, superoxide anion (O_2^-), singlet oxygen, peroxide and hydroxyl radical. These free radicals are capable of causing strand scission of DNA, resulting antitumor action. This suggests that the screening for antibiotics producing such radicals will lead to the finding of new antitumor antibiotics. Paraquat (methylviologen, 1,1′-dimethyl-4,4′-bipyridinium dichloride), which increases intracellular production of superoxide anion in microorganism[4], increases the production of superoxide dismutase in cells[5,6]. We confirmed that the content of superoxide dismutase in *Escherichia coli* was increased by induction with paraquat. We also confirmed that the treatment with paraquat enhanced levels of defense against active oxygen species such as singlet oxygen[7]. We further examined the effect of paraquat on the susceptibility of bacteria to antibiotics producing active oxygen species and devised an agar plate method. For this study, *Bacillus subtilis* PCI219, *Escherichia coli* B, and *Mycobacterium smegmatis* 607 were used. On the basis of the fact that *rec*$^-$ mutant was more susceptible than *rec*$^+$ strain to agents which interacted with DNA[8], we also used *Bacillus subtilis* GSY1028 (*recB*$_2$, *trpC*$_2$, *metB*$_4$) and *Escherichia coli* K12 AR20 (*recA1*, *recB21*, *thi*) as test organisms. These strains were supplied kindly by Prof. H. SAITOH of the Institute of Applied Microbiology of University of Tokyo and Prof. G. TAMURA of the Department of Agricultural Chemistry of University of Tokyo

All strains were grown in Trypticase soy-yeast extract (TSY) medium containing 3% Trypticase broth (BBL) and 0.5% yeast extract (Oriental). Tryptophan (20 μg/ml), methionine (20 μg/ml) and thiamine hydrochloride (1 μg/ml) were supplemented according to demand of test organisms. Test strains cultured overnight at 37°C with shaking were suspended in a prewarmed medium. Paraquat (Sigma Chemical Co.) was added to the suspensions by dilution from sterile stock solutions to obtain 0.3 mM for *B. subtilis* PCI219, *E. coli* B and *Mycobacterium smegmatis* 607 and 0.2 mM for *B. subtilis* GSY1028 and *E. coli* K12 AR20.

A 10 ml of the medium containing a test organism in TSY-2% agar medium was poured into a plastic plate (9 cm in diameter) and solidified at room temperature. Paper discs containing samples were placed on duplicates of plates; the one of them was free of paraquat and the other contained paraquat at the concentration which showed the minimal cytotoxicity. After incubation overnight at 37°C, the difference between inhibition diameters of the two plates was measured.

The data are shown in Table 1. The resistant pattern of 5 test strains grown in the presence of paraquat were the same with all the antibiotics producing oxygen radicals such as bleomycin (HCl), bleomycin (Cu), phleomycin, tallysomycin A, streptonigrin, daunomycin, adriamycin, neocarzinostatin and chartreusin. Furthermore, the difference between inhibition diameters in paraquat-containing and non-containing assay plates was amplified in the case of *rec*$^-$ strain in comparison with *rec*$^+$ strain. In cases of auromomycin, chromomycin A_3 and actinomycin D which interact with DNA and show stronger inhibition against *rec*$^-$ strain than against *rec*$^+$ strain, the inhibition diameters of these antibiotics were not influenced by the addition of paraquat into the assay plate. Mitomycin C, which was known to make not only the cross-linking linkage with DNA[9], but also cause single strand scission[10], was very weak in producing the difference of the inhibition diameters.

The drugs which do not interact with DNA such as chloramphenicol, penicillin G, tetracycline, angolamycin and puromycin produced no difference of inhibition diameters depending on the presence or absence of paraquat.

Saframycin A, which preferentially blocked RNA synthesis[11], did not produce the difference. A few exception was observed in cases of some aminoglycoside antibiotics, that is, inhibition diameter was produced in cases of kanamycin and streptomycin. But, in this case, the inhibition diameter was not amplified by *rec*$^-$ strain. Whereas, other aminoglycoside antibiotics such as 3′,4′-dideoxykanamycin B (dibekacin) and gentamicin did not show the differential activity between paraquat-containing and non-containing conditions. To explain this phenomenon, it can

Table 1. Antimicrobial activity of various agents either in the presence or absence of paraquat.

Antibiotic tested (μg/disc)**		Decrease in zone of inhibition (mm) by paraquat*				
		B. subtilis PCI219	*B. subtilis* GSY1028 (*recB2, trpC2, metB4*)	*E. coli* B	*E. coli* K12 AR20 (*recA1, recB21, thi*)	*Mycobacterium smegmatis* 607
Bleomycin·HCl	(5)	5.5	8.0	4.0	9.0	2.5
	(2.5)	3.8	4.5	1.5	8.5	5.5
Phleomycin	(5)	5.0	8.0	4.0	8.5	5.5
	(2.5)	5.5	8.5	3.5	8.3	3.0
Bleomycin·Cu	(5)	3.3	8.5	5.5	8.5	5.0
	(2.5)	4.5	6.5	6.0	10.5	3.0
Tallysomycin A	(0.5)	7.0	12.0	8.5	11.5	0.5
Streptonigrin	(0.25)	9.5	11.5	7.5	8.8	nd***
Daunomycin	(5)	3.5	11.5	nd	nd	4.5
Adriamycin	(5)	5.0	7.0	nd	nd	2.0
Neocarzinostatin	(5)	5.0	6.5	3.0	8.5	0
Chartreusin	(5)	0	2.5	nd	nd	nd
Mitomycin C	(0.25)	0	0	4.5	1.0	0.5
Auromomycin	(0.5)	0.5	0	nd	nd	nd
Chromomycin A_3	(1)	0	0	nd	nd	nd
Actinomycin D	(1)	0	0	nd	nd	nd
Chloramphenicol	(2.5)	0	0	0	0	nd
Penicillin G	(2.5)	0	0	nd	nd	nd
Saframycin A	(0.5)	0.5	0	nd	nd	nd
Tetracycline	(0.25)	0	0	nd	nd	0
Angolamycin	(5)	0	0	nd	nd	nd
Puromycin	(5)	0	0.5	nd	nd	nd
Kanamycin	(2.5)	4.5	5.5	4.0	2.5	nd
Streptomycin	(2.5)	6.0	2.5	4.0	4.0	8.0
Dibekacin	(2.5)	0	0	0	0	0.5
Gentamicin	(0.25)	1.0	1.2	1.5	1.0	nd

* The difference in diameter of the inhibitory zone of duplicates of assay plates, the one was free of paraquat and the other was supplemented with paraquat (resistant state), was measured by paper disc-diffusion method.

** Figures in parentheses indicate the amounts of antibiotics used in the assay system.

*** nd; Not determined because of its weak antimicrobial activity against the test strains.

be speculated that the reduction of a efficacious concentrations of aminoglycoside antibiotics, which have 3′-hydroxyl group in their structures such as streptomycin and kanamycin, was brought about by the formation of irreversible complex with superoxide dismutase, whose intracellular level had been increased by paraquat, as previously described[12], and the 3′-hydroxyl group may be necessary to bind to the superoxide dismutase. In addition, we investigated whether this assay system is actually useful in detecting oxygen radicals-generating antibiotics. Culture filtrates were, therefore, tested by the paper disc method described above. From culture filtrates selected by this screening, cycloserine, a well-known antibiotics, was isolated.

To our surprise, we found that cycloserine not only produces oxygen radicals but also interacts with DNA molecules (data will be published elsewhere).

In conclusion, the assay system were reported together with the test against *rec*$^-$ strain is useful to find oxygen radicals-generating antibiotics.

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YUZURU MATSUDA
MIKIO KITAHARA
KENJI MAEDA
HAMAO UMEZAWA

Institute of Microbial Chemistry
3-14-23 Kamiosaki, Shinagawa-ku,
Tokyo 141, Japan

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References

1) CONE, R.; S. K. HASAN, J. W. LOWN & A. R. MORGAN: The mechanism of the degradation of DNA by streptonigrin. Can. J. Biochem. 54: 219~223, 1976
2) SOMEYA, A. & N. TANAKA: DNA strand scission induced by adriamycin and aclacinomycin A. J. Antibiotics 32: 839~845, 1979
3) LOWN, J. W.; S.-K. SIM, K. C. MAJUMDAR & R.-Y. CHANG: Strand scission of DNA by bound adriamycin and daunomycin in the presence of reducing agents. Biochem. Biophys. Res. Comm. 76: 705~710, 1977
4) HASSAN, H. M. & I. FRIDOVICH: Intracellular production of superoxide radical and of hydrogen peroxide by redox active compounds. Arch. Biochem. Biophys. 196: 385~395, 1979
5) HASSAN, H. M. & I. FRIDOVICH: Regulation of superoxide dismutase synthesis in *Escherichia coli*: Glucose effect. J. Bacteriol. 132: 505~510, 1977
6) HASSAN, H. M. & I. FRIDOVICH: Regulation of the synthesis of superoxide dismutase in *Escherichia coli*. J. Biol. Chem. 252: 7667~7672, 1977
7) MATSUDA, Y.; M. KITAHARA, K. MAEDA & H. UMEZAWA: Correlation between level of defense against active oxygen in *Escherichia coli* K12 and resistance to bleomycin. J. Antibiotics 35: 931~933, 1982
8) MATSUI, I.; S. MURAKAWA & T. TAKAHASHI: Attempt of microbial mutagen screening with Rec-assay; isolation of chartreusin. Agric. Biol. Chem. 44: 919~920, 1980
9) LOWN, J. W. & A. BEGLEITER: Studies related to antitumor antibiotics. V. Reactions of mitomycin C with DNA examined by ethidium fluorescence assay. Can. J. Biochem. 54: 110~119, 1976
10) UEDA, K.; J. MORITA & T. KOMANO: Induction of single strand scission in bacteriophage ϕX174 replicative form I DNA by mitomycin C. J. Antibiotics 34: 317~322, 1981
11) ISHIGURO, K.; S. SAKIYAMA, K. TAKAHASHI & T. ARAI: Mode of action of saframycin A, a novel heterocyclic quinone antibiotic. Inhibition of RNA synthesis *in vivo* and *in vitro*. Biochemistry 17: 2545~2550, 1978
12) BRITTON, L. & I. FRIDOVICH: Streptomycin: Irreversible association with superoxide dismutase. Arch. Biochem. Biophys. 191: 198~204, 1978