VOL. 52, NO. 1, JAN. 1999 THE JOURNAL OF ANTIBIOTICS pp. 65 — 67

Inhibitory Effect of Antimicrobial Agents on Alginate Production in *Pseudomonas aeruginosa*

TAKAYUKI HOSOYAMA, TAKEHIRO YUGE†, SENJI TAKAHASHI, TOMOO SUZUKI†, TOYOJI OKUBO††, SHIZUKO IYOBE†† and AKIRA NAKAGAWA*

Department of Biosciences, Teikyo University, 1-1 Toyosatodai, Utsunomiya 320-8551, Japan
† Department of Applied Biochemistry, Utsunomiya University, 350 Mine-machi, Utsunomiya 321-8505, Japan
†† Laboratory of Drug-resistance in Bacteria, Gunma University, School of Medicine, 3-39-22 Showa-machi, Maebashi 371-8513, Japan

(Received for publication September 24, 1998)

The biofilm formed in local lesions by *Pseudomonas aeruginosa* is mainly composed of alginate and considered to be a barrier against host defense mechanisms and chemotherapeutic agents, causing serious and chronic infections[1~3]. Alginate is a polymer consisting of β-D-mannuronate and its epimer α-L-guluronate, and *P. aeruginosa* β-D-mannuronate has been characterized to be *O*-acetylated at a higher rate in comparison with others[4,5]. Guanosine diphosphomannose dehydrogenase (GMD) is characterized as the key enzyme in alginate formation in *P. aeruginosa*[6~8]. Therefore, substances that specifically inhibit GMD activity are expected to be effective therapeutic agents against biofilm-forming *P. aeruginosa*. We reported that morphological alteration of mucoid to non-mucoid in *P. aeruginosa* and inhibition on GMD activity might be an useful method to screen alginate biosynthetic inhibitors from microbial metabolites[9]. In this paper we describe mucoid to non-mucoid alteration, inhibition of alginate production, and GMD inhibition by clinically used antibacterial agents and their relationship.

The *P. aeruginosa* PAM38 which constitutively formed mucoid colonies was used for the determination of the minimum concentration inducing non-mucoid alteration (MCINA) and for measurement of alginate production. Minimum inhibitory concentration (MIC) and MCINA were examined by an agar dilution method. Alginate production was estimated by HPLC according to the method of TOYODA *et al.*[10] and inhibitory rate of alginate formation was expressed by the percent ratio of HPLC peak area. The enzyme GMD was purified from a culture of *P. aeruginosa* strain PAM17 which produced the enzyme in large amounts by the method of ROYCHOUDHURY[8] with some modifications. The following antibacterial agents were used: erythromycin A (EM-A), clarithromycin (CAM), azithromycin (AZM), oleandomycin (OL), josamycin (JM), gentamicin (GM), clindamycin (CLDM) and chloramphenicol (CP). Penicillic acid (PCA) was found in the screening system using morphological alteration of mucoid to non-mucoid in *P. aeruginosa*[9].

In order to know whether the decrease of alginate production resulted from growth inhibition by the antibacterial agents used, the amounts of alginate produced under various concentrations (1/4, 1/16, 1/64 and 1/256 MIC) of the agents were estimated by HPLC analysis. Viable cell counts in all the cultures were not affected under the condition of 1/4 MIC or less of the agents, indicating that a decrease in alginate formation is not concerned by bacterial viability. Table 1 summarizes MIC values of antibacterial agents against *P. aeruginosa*, their inhibitory effects on alginate production, MCINA and GMD activity. As shown in Table 1, low concentration of AZM (0.78 μg/ml), CP (0.78 μg/ml) and PCA (6.25 μg/ml), which corresponds to 1/256 MIC showed strong inhibition of alginate formation, 75, 94 and 85%, respectively. EM-A, CAM, OL and CLDM showed the inhibition dose-dependently. On the other hand, the inhibitory activity by JM and GM was lower level.

As it was predicted that a decrease in alginate formation might result from inhibition of GMD activity, we examined the relation between MCINA activity and inhibitory effect on GMD by antibacterial agents. Table 1 summarizes MCINA for PAM38 strain and 50% inhibitory concentrations (IC_{50}) for GMD activity by agents. As expected, inducibility of non-mucoid alteration by each agent except for JM related to the inhibition of alginate formation. The non-mucoid alteration of GM could not be determined because of its antibacterial activity against *P. aeruginosa*. AZM and CP showed a low MCINA (0.78 μg/ml). Inhibitory activities on mucoid formation of EM-A and CAM were relatively low with MCINA 6.25 μg/ml, but that of OL, JM and CLDM were weak (MCINA 100 μg/ml). On the contrary, the IC_{50} for GMD activity were 1.1 mM for EM-A and 0.6 mM for AZM in which their inhibitory activities

Table 1. The effects on MIC, inhibition of alginate production, MCINA, and inhibitory activity for GMD of *P. aeruginosa* by antimicrobial agents and penicillic acid.

Compounds	MIC (μg/ml)	Inhibition of alginate production (% of control)				MCINA (μg/ml)	50% Inhibition of GMD (mM)
		1/4 MIC	1/16 MIC	1/64 MIC	1/256 MIC		
EM-A	200	90	87	48	22	6.25	1.1
CAM	200	72	67	58	50	6.25	>1.0
AZM	200	86	76	73	75	0.78	0.6
OL[a]	>1600	94	83	53	52	100	>2.0
JM	800	45	52	32	38	100	>1.0
CLDM[a]	>1600	92	89	70	40	100	>2.0
GM	6.25	17	28	32	12	—	>2.0
CP	100	93	96	97	94	0.78	>2.0
PCA[a]	>1600	91	89	89	85	6.25	2.4

[a] Inhibitory effect on alginate production of OL, CLDM and PCA was examined as MIC; 1600 μg/ml.

were not strong. The other agents, OL, JM, CAM, CP, CLDM and GM had almost no GMD inhibitory activity. The inhibitory concentrations above 1.0 mM of CAM and JM could not be determined because of their low solubility in the solvent. No detectable inhibitory activity of CP and PCA on GMD were observed, although the agent strongly inhibited alginate production. The inhibition on alginate production and non-mucoid alteration did not always correlate to the inhibitory activity on GMD, indicating that the inhibition on alginate production of CP and PCA[11] might result from such mode of actions as inhibition of protein synthesis and cytotoxicity, respectively. KOBAYASHI *et al.*[12] have reported that the inhibitory effect of EM-A and AZM on GMD is related to a decrease on alginate production. Our results suggest that none of EM, CAM, AZM and CP would be a specific inhibitor of GMD, although these agents resulted in the suppression of alginate production. TATEDA *et al.*[13] have reported that sub-MICs of AZM affect viability and protein synthesis in several strains of *P. aeruginosa*, due to the excellent permeability through the bacterial membrane. Although the possibility remains to be examined, it can be predicted that the high non-mucoid alteration but a weak inhibition of EM-A and AZM on GMD activity, in comparison with OL and JM seems to be due to the difference of permeability through the bacterial membrane, not a direct inhibition on GMD and may be attributable to the suppression of alginate-biosynthesis-related enzyme systems resulting from inhibition of protein synthesis in *P. aeruginosa*.

ELLOUMI *et al.*[14] reported that structural analogues of GDP mannose inhibited GMD activity, suggesting that the inhibitors of alginate biosynthesis should be detectable in certain substances relating to enzymatic reactions. So far, there is no report on an inhibitor on alginate biosynthesis from natural products. In order to screen a specific inhibitor on alginate biosynthesis, it needs to examine the effect on other mode of action, in addition to inhibitory activity on GMD activity and mucoid formation.

Acknowledgments

The authors wish to thank Miss M. MAKINO for an excellent technical assistance. The authors are grateful to Dr. M. NAKANO for stimulating discussion and advice.

References

1) COSTERTON, J. W.; K.-J. CHENG, G. G. GEESEY, T. I. LADD, J. C. NICKEL, M. DASGUPTA & T. J. MARRIE: Bacterial biofilms in nature and disease. Ann. Rev. Microbiol. 41: 435~464, 1987
2) HOYLE, B. D. & J. W. COSTERTON: Bacterial resistance to antibiotics: The role of biofilms. Prog. Drug. Res. 37: 91~105, 1991
3) TANIMOTO, H.: A review of the recent progress in treatment of patients with diffuse panbronchiolitis associated with *Pseudomonas aeruginosa* infection in Japan. Antibiot. Chemother. 44: 94~98, 1991
4) EVANS, L. R. & A. LINKER: Production and characterization of the slime polysaccharide of *Pseudomonas aeruginosa*. J. Bacteriol. 116: 915~924, 1973
5) SKJÅK-BRÆK, G.; H. GRASDALEN & B. LARSEN: Monomer sequence and acetylation pattern in some bacterial alginates. Carbohydr. Res. 154: 239~250, 1986
6) TATNELL, P. J.; N. J. RUSSELL & P. GACESA: GDP-mannose dehydrogenase in the key regulatory enzyme in alginate biosynthesis in *Pseudomonas aeruginosa*: evidence from metabolite studies. Microbiol. 140: 1745~1754, 1994
7) PUGASHETTI, B. K.; L. VADAS, H. S. PRIHAR & D. S. FEINGOLD: GDPmannose dehydrogenase and biosynthe-

sis of alginate-like polysaccharide in a mucoid strain of *Pseudomonas aeruginosa*. J. Bacteriol. 153: 1107~1110, 1983

8) ROYCHOUDHURY, S.; T. B. MAY, J. F. GILL, S. K. SINGH, D. S. FEINGOLD & A. M. CHAKRABARTY: Purification and characterization of guanosine diphospho-D-mannose dehydrogenase. J. Biol. Chem. 264: 9380~9385, 1989

9) NAKAGAWA, A.; T. HOSOYAMA, K. CHUBACHI, S. TAKAHASHI, T. OKUBO & S. IYOBE: A search for *Pseudomonas* alginate biosynthesis inhibitors from microbial metabolites. J. Antibiotics 50: 286~288, 1997

10) TOYODA, M.; C. YOMOTA, Y. ITO & M. HARADA: High performance liquid chromatographic method for the determination of sodium alginate in foods. J. Food Hyg. Soc. Jpn. 26: 189~194, 1985

11) BIRKINSHAW, J. H.; A. E. OXFORD & H. RAISTRICK: Studies in the biochemistry of microorganisms. XLVIII. Penicillic acid, a metabolic product of *Penicillium puberulum* bainier and *P. cyclopium* westling. Biochem. J. 30: 394~411, 1936

12) KOBAYASHI, H.: Airway biofilm disease: clinical manifestations and therapeutic possibilities using macrolides. J. Infect. Chemother. 1: 1~15, 1995

13) TATEDA, K.; Y. ISHII, T. MATSUMOTO, N. FURUYA, M. NAGASHIMA, T. MATSUNAGA, A. OHNO, S. MIYAZAKI & K. YAMAGUCHI: Direct evidence for antipseudomonal activity of macrolides: Exposure-dependent bactericidal activity and inhibition of protein synthesis by erythromycin, clarithromycin and azithromycin. Antimicrob. Agents Chemother. 40: 2271~2275, 1996

14) ELLOUMI, N.; B. MOREAU, L. AGUIAR, N. JAZIRI, M. SAUVAGE, C. HULEN & M. L. CAPMAU: Inhibitors of GDP-mannose dehydrogenase of *Pseudomonas aeruginosa* mucoid strains. Eur. J. Med. Chem. 27: 149~154, 1992