Communications to the Editor

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INCREASED PERMEABILITY OF THE OUTER MEMBRANE OF *Escherichia coli* INDUCED BY THE DIMER IN COMPOUND 48/80

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The following two facts show that the dimer in compound 48/80, bis[2-methoxy-5-(2-methylaminoethyl)phenyl]methane dihydrochloride, increases the permeability of the outer membrane of *Escherichia coli*. First, addition of the dimer to the cell suspension stimulated the uptake of tetraphenylphosphonium cations. Second, the synergistic effect of the dimer on the action of the antibiotics fusidic acid or novobiocin inhibited the growth of the organism.

KEYWORDS — compound 48/80; polycation; dimer; outer membrane; membrane permeability; TPP^+ uptake; growth inhibition test; structure-activity relationship; gram-negative bacteria; *Escherichia coli*

Recent studies[1-4] have shown that basic polypeptides such as polylysine, protamine, and polymyxin analogs render the outer membrane of gram-negative bacteria permeable to several drugs. These basic polypeptides bind lipopolysaccharide (LPS) molecules in the outer membrane,[5-7] and this binding is considered to trigger disruption of the permeability barrier of the membrane.[3-7] It has also been reported, however, that polycations with a few basic charges do not disrupt the barrier function of the outer membrane,[2] though these cations can bind isolated LPS.[2,8] The structure-activity relationship of the increase in permeability remains obscure at present. We compared the action of polycations with that of ethylenediamine tetraacetate (EDTA). It is well known that treating bacteria with EDTA disrupts the permeability barrier of the outer membrane.[9] EDTA eliminates divalent cations at the binding site of LPS, apparently triggering disruption of the barrier function. We assumed that a dication could also increase permeability of the outer membrane, if it had an appropriate molecular structure. It is probable that divalent cations at binding sites are removed efficiently by dications, leading to disruption of the outer membrane structure. In the course of investigating the action of polycations on *Escherichia coli* cells, we found a novel dication having the ability

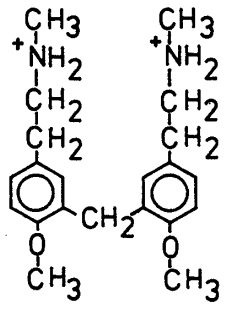

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Chart 1

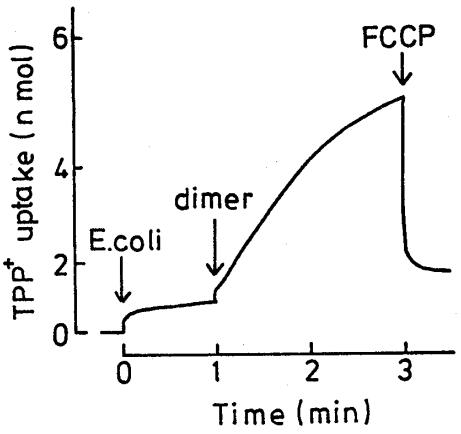

Fig. 1. The Uptake of TPP^+ after Addition of the Dimer in 48/80

Assay mixtures consisted of 1.0 ml of 50 mM 4-morpholinepropanesulfonic acid-tris(hydroxymethyl)aminomethane (pH 7.2), 100 mM choline chloride, 10 mM sodium lactate, and 10 μM TPPCl. At the first arrow, 50 μl of a cell suspension (0.5 mg cell protein) was added. The second arrow indicates the time when 50 μl of the dimer in 48/80 (final concentration: 50 μg/ml) was added, and at the third arrow FCCP (1 μl of ethanol solution, final concentration: 1 μM) was added. Measurements were made at 28°C.

to disrupt the barrier function of the outer membrane. Here we report that the dimer in compound 48/80 (1), bis[2-methoxy-5-(2-methylaminoethyl)phenyl]methane dihydrochloride,[10] can increase the permeability of the *E. coli* outer membrane.

The bacterial strain used in this study was *E. coli* W3110, a derivative of K12. Cells were grown at 37°C in a minimal salt medium supplemented with 1% polypeptone.[3,4] Preparation of the cells was the same as described previously.[3,4]

We measured the uptake of tetraphenylphosphonium cation (TPP^+) to test whether the dimer in 48/80 can disrupt the permeability barrier of the outer membrane. It is known that TPP^+, a lipophilic cation, diffuses passively across the membrane, depending on the membrane potential.[11,12] Such diffusion does not occur efficiently in gram-negative bacteria, due to the presence of the outer membrane.[13] Disruption of the permeability barrier of the outer membrane stimulates the uptake of TPP^+.[3,4] Figure 1 shows an increase in the uptake of TPP^+ upon addition of the dimer in 48/80. At time zero, *E. coli* suspension was added to the buffer solution containing 10 μM TPP^+ and 10 mM sodium lactate. A small TPP^+ uptake was observed, probably due to adsorption to cells. When the dimer was added to this suspension, the uptake of TPP^+ was stimulated, indicating that the dimer disrupted the barrier function of the outer membrane. Then we added an uncoupler. carbonyl cyanide

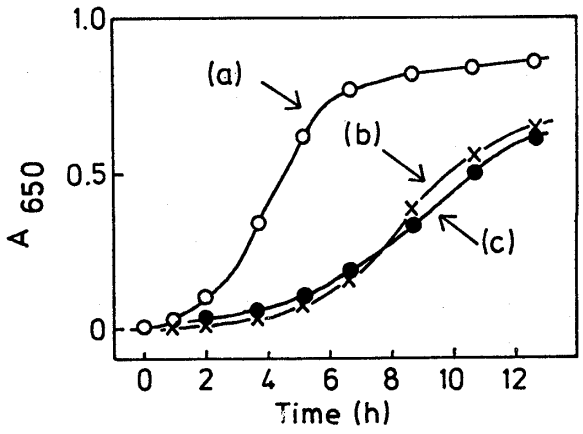

Fig. 2. Growth Inhibition of Cells Induced by the Synergism of the Dimer in 48/80 Acting with Antibiotics

Growth curves were determined by a conventional method using a liquid medium. The medium containing a minimal salt supplemented with 1% polypeptone and 25 μg/ml of the dimer in 48/80 was inoculated with 10^7 cells/ml. After incubation for 5 min at 37°C, fusidic acid or novobiocin (concentration of each: 25 μg/ml) was added, and incubation was continued at 37°C. Each point on the growth curves was determined by measuring the absorbance at 650 nm (A_{650}). (a) control without drugs, (b) with the dimer and fusidic acid, (c) with the dimer and novobiocin.

p-trifluoromethoxyphenylhydrazone (FCCP), to the cell suspension. The FCCP abolished the membrane potential causing the efflux of the accumulated TPP^+.

To obtain further evidence on the dimer-induced disruption of the barrier function, we examined the synergistic effects of the dimer on the action of the antibiotics fusidic acid and novobiocin. It is known that both antibiotics are less effective on gram-negative bacteria.[1,14] The barrier function of the outer membrane prevents penetration of the antibiotics into cells. We measured the growth of cells in the presence of the dimer with each of the antibiotics (Fig. 2). A control experiment without any addition of drugs is shown in Fig. 2(a). When both the dimer and fusidic acid were added to the cell suspension, the growth rate decreased (Fig. 2(b)). Of course, it was confirmed that the addition of the dimer or fusidic acid alone did not affect the growth rate of the cells. Cell growth was also inhibited by the synergistic effect of the dimer on the action of novobiocin (Fig. 2(c)).

These results show that the dimer in 48/80 disrupted the barrier function of the outer membrane to enhance the permeation of the drugs. Further studies are now in progress to clarify the structure-activity relationship required for the permeability increase.

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