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Sensitization of *E. coli* to the Serum Bactericidal System and to Lysozyme by Ethyleneglycoltetraacetic Acid (38028)

CHARLES S. BRYAN
(Introduced by Roger M. Des Prez)
Veterans Administration Hospital and the Department of Medicine, Vanderbilt University, Nashville, Tennessee 37203

The serum bactericidal system is lethal to some gram-negative bacteria by the combined action of complement and lysozyme against the cell wall. Complement is considered to damage the outer lipopolysaccharide portion of the cell wall, while lysozyme acts on the inner peptidoglycan polymer (1). The basis for the sensitivity of some strains and the resistance of others to this system is uncertain.

Some strains of gram-negative bacteria which are ordinarily resistant to this system can be rendered sensitive to the effects of both lysozyme (2) and complement (3) by exposure to ethylenediaminetetraacetic acid (EDTA). This effect is attributed to alteration of the cell wall by this chelator. Several lines of evidence suggest that the critical divalent cation required for maintaining the structural integrity of the cell wall, and removed therefrom by EDTA, is magnesium (4, 5).

EDTA has approximately equal avidity for Ca^{2+} and Mg^{2+} ions. Ethyleneglycoltetraacetic acid (EGTA) has the property of having over 100,000 times greater avidity for calcium than for magnesium (6). The addition of $MgCl_2$ in equimolar quantity to a solution of EGTA forms Mg EGTA, restoring the Mg^{2+} concentration while moderately reducing the efficiency of Ca^{2+} chelation (Table I). Thus, if Mg^{2+} is the critical divalent cation for maintaining the structural integrity of the *E. coli* cell wall, it might be anticipated that EGTA would show some ability to sensitize bacteria to the effects of complement and lysozyme, whereas Mg EGTA would not show this property. The present experiments test this hypothesis.

TABLE I. Approximate Ca^{2+} and Mg^{2+} Concentrations in Serum and Hank's Balanced Salt Solution Containing Chelators.

Chelator	Mg^{2+} (*M*)	Ca^{2+} (*M*)
None	10^{-3}	10^{-3}
10 m*M* EDTA	10^{-10}	10^{-12}
10 m*M* EGTA	10^{-6}	10^{-11}
10 m*M* Mg EGTA	10^{-3}	10^{-10}

Materials and Methods. Solutions of 100 m*M* EGTA were prepared as previously described from this laboratory (6). To prepare 100 m*M* solutions of Mg EGTA, equimolar quantities of $MgCl_2$ were added to 100 m*M* EGTA solutions. *E. coli* ATCC 25922, a strain relatively resistant to the serum bactericidal system (no significant killing observed after incubation in 90% fresh serum at 37° for 4 hr), was used as the test organism and was maintained on trypticase soy (TS) agar slants. Prior to use, cultures were grown in TS broth at 37°. Serum was obtained from healthy volunteers and stored at −70° for 1 week or less prior to use. Egg white lysozyme (Worthington Biochemical Corp., Freehold, NJ) was dissolved in Hank's balanced salt solution (HBSS) containing 0.1% gelatin. When necessary, 0.2 *M* NaOH was added to solutions of HBSS with chelators to restore pH to approximately 7.0.

Assays for bactericidal activity were performed in 75 × 12 mm plastic tubes (Falcon Plastics, Oxnard, CA) containing 1.8 ml of fresh or heated (56°, 30 min) serum or HBSS with 0.1% gelatin and 0.2 ml of chelator solution or normal saline. Bacteria were grown at 37° in TS broth prior to use. Assays for susceptibility to the serum bactericidal system were done with approximately 3 × 10^3 bacteria/ml, obtained from 3-hr cultures. Assays for susceptibility to lysozyme in HBSS were done with approximately 3 × 10^5 bacteria/ml from 18-hr cultures. In both assay systems, the tubes were rotated with gentle end-over-end tumbling on a tube rotator at 37° for 4 hr, following which bacterial survival was determined from TS agar pour plates made from appropriate dilutions of the assay mixtures.

Levels of significance were determined by Student's t test.

Results and Discussion. In assays for susceptibility of organisms to the serum bactericidal system (Fig. 1), fresh serum with EGTA showed significant killing compared to heated serum with EGTA ($P <$ 0.001), while fresh serum with Mg EGTA did not. The optimum Mg^{2+} concentration for the serum bactericidal system is considered to be approximately that of the physiologic range (10^{-3} *M*) (7, 8). That killing occurred in EGTA serum (Mg^{2+} concentration of 10^{-6} *M*) but not in Mg EGTA serum (Mg^{2+} concentration of 10^{-3} *M*) thus seems contrary to what would be expected. A suggested explanation is that damage to the cell wall by EGTA due to its Mg^{2+}-chelating property rendered the organisms sensitive to the serum bactericidal system. Such damage did not occur in Mg EGTA serum, since it provides a physiologic concentration of Mg^{2+} ion.

In assays for susceptibility of organisms to lysozyme in HBSS, EGTA significantly reduced bacterial survival when a high concentration of lysozyme (1000 μg/ml) was used (Fig. 2) ($P < 0.01$). Killing was not present when Mg EGTA or normal saline (control) was used. Prior dialysis of the lysozyme solution against NaCl to re-

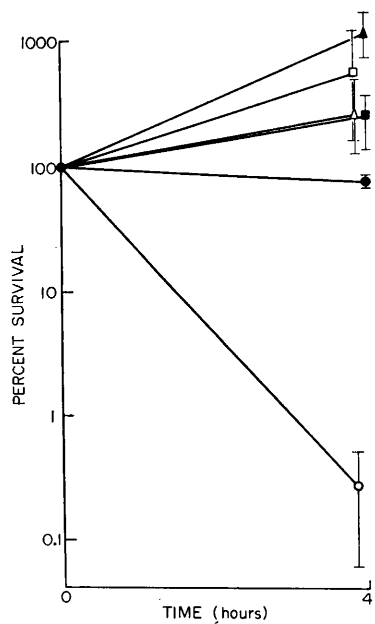

FIG. 1. Effect of 10 m*M* concentrations of EGTA and Mg EGTA, compared to saline, on survival of *E. coli* ATCC 25922 in fresh and heated human serum. Each point represents the mean (±SEM) of three experiments: (○) fresh serum with EGTA, (●) heated serum with EGTA, (□) fresh serum with Mg EGTA, (■) heated serum with Mg EGTA, (△) fresh serum with saline, (▲) heated serum with saline.

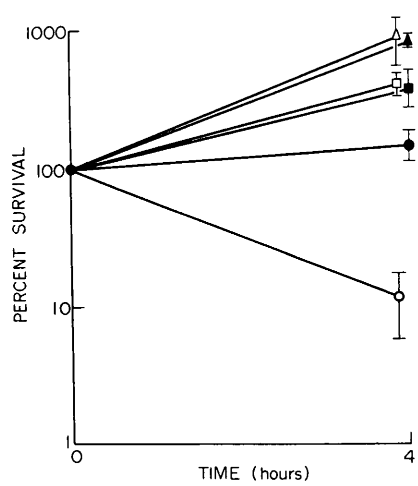

FIG. 2. Effect of 10 m*M* concentrations of EGTA and Mg EGTA, compared to saline, on survival of *E. coli* ATCC 25922 in Hank's balanced salt solution with and without the addition of lysozyme, 1000 μg/ml. Each point represents the mean (±SEM) of five experiments: (○) EGTA and lysozyme present in system, (●) EGTA without lysozyme, (□) Mg EGTA and lysozyme present in system, (■) Mg EGTA without lysozyme, (△) saline and lysozyme present in system, (▲) saline without lysozyme.

move possible cation contaminants did not alter these results. The need for a high concentration of lysozyme to demonstrate killing in the presence of EGTA is possibly due to the relative insusceptibility of gram-negative bacilli to lysozyme in the absence of complement.

Two implications of this study are as follows: First, by comparison of the chelating properties of EGTA and Mg EGTA and their "sensitizing" properties, it is apparent that Mg^{2+} ion plays an important role in maintaining the structural integrity of the cell wall of *E. coli.* Thus, EGTA and its salts might prove useful reagents in the study of the role of divalent cations in bacterial structure and metabolism. Secondly, because of different requirements for Ca^{2+} and Mg^{2+} by the classical and alternate pathways of complement activation, EGTA has been introduced as a reagent for distinguishing between these two pathways (6). The present findings indicate that, when EGTA is present in a system, proper controls should be included to rule out the possibility that the biologic end-point is affected by damage to the target cell by this chelator.

Summary. EGTA, but not Mg EGTA, was found to enhance the effects of the serum bactericidal system and of lysozyme on the survival of *E. coli* ATCC 25922. These observations are consistent with the hypothesis that Mg^{2+} is the critical divalent cation for maintaining the structural integrity of the *E. coli* cell wall.

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