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Quantitative Studies on Spheroplast Formation by the Complement System and Lysozyme on Gram Negative Bacteria.* (31915)

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Gram negative bacteria of the genera *Vibrio, Salmonella, Shigella, Escherichia, Brucella* and *Hemophilus* may be killed by the complement (C′) system(1). Some of the members of these groups, particularly rough forms, are extremely sensitive to small amounts of fresh normal serum, which serves as a source of both natural antibody and C′. Other organisms are not killed unless antiserum is present in addition to C′, and a third group, under the usual experimental conditions, is refractory to C′ despite sensitization by antiserum(2). Among the Enterobacteriaceae, resistance to normal serum has been associated with the capsular (K) antigenic content of the organism(3) and, more significantly, from a biomedical point of view, with the virulence of the organism (4).

Although the bactericidal and bacteriolytic reactions mediated by serum substances have not generally been distinguished, recent observations have indicated that the bactericidal reaction requires antibody and C′, whereas the lysis of the killed cells also requires the enzyme lysozyme(5,6). C′ activity is not unique in rendering cells of gram negative bacteria susceptible to lysozyme attack since starved cells at an abnormal pH(7) or treatment with polymyxin B sulfate(8) will accomplish this result. Obviously different mechanisms may lead to cell lysis by lysozyme.

Bacteria rendered non-viable by the C′ system are not grossly distorted, but are lysed only upon subsequent addition of lysozyme, or in a stabilizing milieu, converted to spheroplasts(5). The quantitative relationships between the susceptibility of an organism to C′ and to lysozyme have not been determined. It was not known whether cells killed by C′ derived from relatively sensitive organisms were equally or differently sensitive to lysozyme. Consequently, cells of organisms

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of varying sensitivity to C′ were subjected to its action and the amount of lysozyme required for conversion of those cells to spheroplasts was determined(9).

Materials and methods. Salmonella typhi strains Ty2, Watson, O901 and R-2, *Escherichia coli* strain B, and *Vibrio cholerae* strain Inaba 20-A-10 were obtained from the culture collection of the Walter Reed Army Institute of Research, Washington, D.C. and maintained on meat extract agar plates. For the preparation of antiserum, rabbits were injected intravenously with about 10^8 cells of one of the organisms and bled one week later. Removal of lysozyme from serum was accomplished by absorption with bentonite (Volclay, American Corp.). The bentonite was washed 10 times with distilled water, 4 mg of the washed material added for each ml of serum, and the suspension kept at 4°C for 10 minutes and centrifuged. This procedure removed lysozyme below a detectable limit (0.1 μg/ml). Fresh guinea pig serum as a C′ source was absorbed additionally at 4°C with washed heat-killed cells of the test organism to remove natural antibody necessary for bactericidal action until 0.5 ml of the serum killed less than 10% of a standardized inoculum. Depending upon the organism used and the sample of guinea pig serum, 2 to 6 absorptions were required for elimination of bactericidal activity. The diluent used in all the tests was 0.85% NaCl and 0.063% $MgCl_2 \cdot 6H_2O$. Bactericidal activity was measured by a photometric growth assay procedure and lysis equated with spheroplast formation in hypertonic solutions. Two sets of tubes were required with appropriate amounts of antiserum, C′, diluent and culture containing about 2×10^7 organisms. After 1 hour at 37°C, 5.0 ml of brain heart infusion broth was added for the bactericidal determination which was performed by the quantitative photometric assay(10). To the duplicate set of tubes for the spheroplast assay, 100 μg/ml egg white lysozyme (Worthington Biochemical Corp., Freehold, N. J.), representing an excessive amount, was added, and 0.6 ml of spheroplast stabilizing medium consisting of an aqueous solution of 25% sucrose and 5% $MgSO_4$. After 60 minutes the reaction was stopped by addition of 0.5 ml of formaldehyde (37% solution). The percentage conversion to spheroplasts was calculated after counting 200 organisms using a phase contrast microscope. When antiserum was being titrated, the logarithm of the different amounts was plotted against the percentages of spheroplasts converted to probits and the 50% serum endpoint and slope of the dose-response relationship determined with the aid of logarithmic probability paper such as Keuffel and Esser No. 358-22(11). Results have been expressed in terms of the reciprocal of this endpoint or titer.

The spheroplast assay with limiting amounts of lysozyme was performed by first treating the standard inoculum of 1.8×10^7 cells with that amount of antiserum and complement giving over 98% killing as determined previously by the photometric bactericidal assay. After the bactericidal reaction which occurred during incubation of the reactants at 37°C for 60 minutes, limiting amounts of lysozyme ranging from 0.1 μg to 100 μg and stabilizing medium were added to the reaction mixture including the killed organisms. After an additional hour of incubation 0.5 ml of formaldehyde (37% solution) was added to stop the reaction, and the percentage of spheroplasts formed was determined microscopically. Control tubes included antiserum alone, complement alone, diluent alone, all with and without lysozyme.

The assays were repeated at least once. The Table and Figures represent results obtained from a single typical assay.

Results. Antisera against a variety of organisms were titrated by the bactericidal and spheroplast assays. The data given in Table I indicate that the results obtained by both assays were in excellent agreement. More detailed graphical representation of the data is presented in Fig. 1. One notes that the lines are of equal but opposite slope and that the combined percentage of spheroplasts and survivors for any particular antiserum amount is equal to 100%. These data suggest that as each organism was killed, it was converted to a spheroplast in the presence of excess lysozyme. Very similar curves were obtained with other test organisms which included *S. typhi*

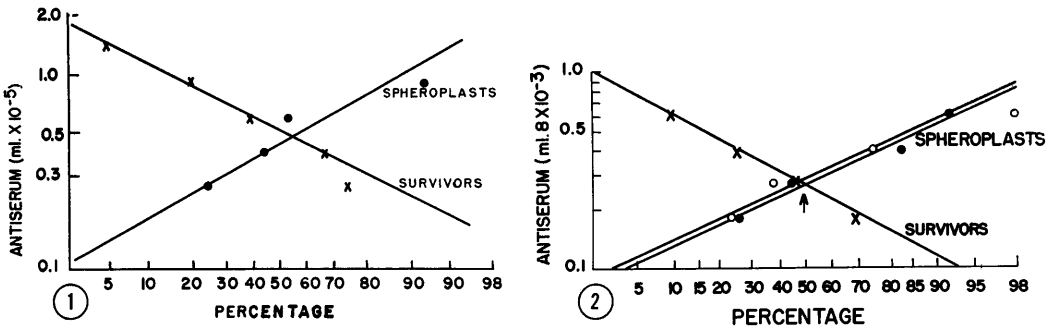

FIG. 1. Antiserum titration by bactericidal and spheroplast assays with complement and lysozyme in excess against *S. typhi* 0901. Ordinate scale, logarithmic; abscissa scale, normal deviate units.

FIG. 2. Antiserum titration by bactericidal and spheroplast assays with antiserum and complement lacking in detectable lysozyme against *V. cholerae* Inaba. The spheroplast assay was performed without lysozyme (closed circles) and with addition of 40 μg of lysozyme (open circles) to each tube. Ordinate scale, logarithmic; abscissa scale, normal deviate units.

strains R-2, Watson, Ty2, and *E. coli* strain B.

Results obtained with *V. cholerae* Inaba indicated that lysozyme, at least in detectable amounts, was not required for spheroplast formation. An attempt was made to remove as much lysozyme as possible by 6 additional absorptions with bentonite of the complement source as well as the antiserum. Using these reagents, the addition of 40 μg of lysozyme to each of the assay tubes did not influence the spheroplast assay (Fig. 2). Moreover, controls such as antiserum and excess lysozyme, complement and excess lysozyme, and complement alone, which did not exert a vibriocidal effect, yielded an insignificant number of spheroplasts (1-3%).

The next experiments were performed using cells killed by the C′ system with lysozyme-free reagents. The amount of lysozyme required for conversion of the C′ killed cells to spheroplasts was then determined. The results given in Fig. 3 indicate conclusively that the amount of lysozyme required is a function of the test organism. Of unusual interest was the marked difference in the dose-response relationship of lysozyme against *S. typhi* Ty2 compared to the other organisms, with slope of 0.4 for the former and approximately 1.4 for the others. This result indicates that a disproportionately larger amount of lysozyme is needed to effect a comparable percentage increase of spheroplast conversion with *S. typhi* Ty2 than with the others. The different slopes may reflect differences in the number of available substrate sites, steeper slopes indicating a relatively greater number of sites. Also noteworthy is the fact that among the *S. typhi* strains tested, Ty2, the most resistant to lysozyme induced spheroplast formation, is highly virulent, relatively resistant to the bactericidal action of normal serum, and contains the largest amount of Vi antigen. Strain Watson, which follows in lysozyme resistance,

TABLE I. Titers of Antisera Against Different Organisms Determined by Bactericidal and Spheroplast Assays with Excess Lysozyme.

Organism	Bactericidal titer*	Spheroplast titer*	% Difference in 50% endpoint titers†	Lysozyme amount, μg
E. coli B	37,000	43,000	7.5	50
S. typhosa 0901	200,000	220,000	4.7	20
S. typhosa Watson	33,000	37,000	5.7	100
S. typhosa R-2	3,100	2,200	17	100
S. typhosa Ty2	49,000	36,000	15	500

* Represents reciprocal of antiserum amount required for killing or spheroplast formation of 50% of test inoculum.

† Percent difference determined by taking the mean of the bactericidal and spheroplast titers and dividing the difference by the mean of the assays.

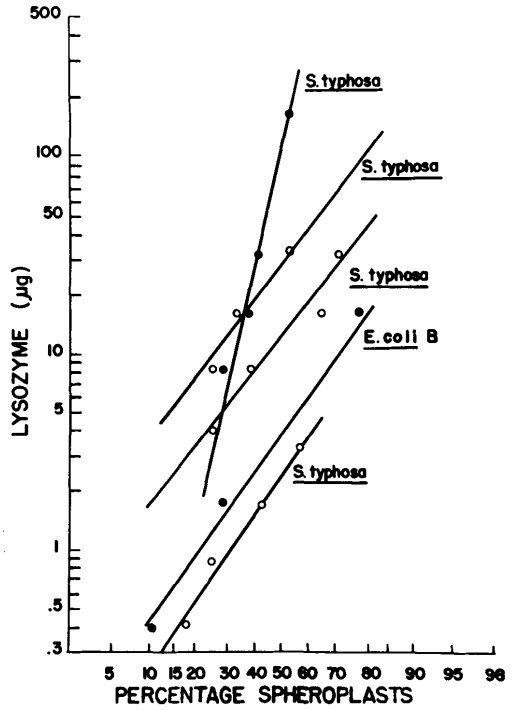

FIG. 3. Lysozyme titration determined by spheroplast formation of complement killed cells of different organisms. Ordinate scale, logarithmic; abscissa scale, normal deviate units.

is less virulent and contains less Vi antigen than strain Ty2, while strains O901 and R-2 contain no detectable Vi antigen(3). Thus, resistance to lysozyme may be associated with the Vi or other K antigens. Finally, *V. cholerae* Inaba did not require lysozyme, or at least less than a detectable amount (0.1 μg) for conversion of its C′ killed cells to spheroplasts. With this organism it seems that the bactericidal reaction may be equated with visible and pronounced lysis.

Discussion and summary. The analyses of the immune bactericidal reaction and of spheroplast formation have indicated, in confirmation of earlier work, a quantitative conversion of C′ killed antibody sensitized cells to spheroplasts by lysozyme. This was shown graphically by plotting the bactericidal data in terms of the percentage of surviving organisms and by a similar plot of spheroplast formation as the percentage of spheroplasts formed in the presence of excess lysozyme. The slopes of the lines representing, respectively, the probit of the percentage of surviving organisms and of spheroplasts, plotted against the logarithm of the antiserum were equal and opposite with the intersection point close to the 50% endpoints (Fig. 1). Sensitized cells of *V. cholerae*, however, were killed and converted to spheroplasts by C′ alone without the addition of lysozyme. Previous workers(12) have also observed protoplast formation, without added lysozyme, resulting from the action of antibody and C′ against *V. cholerae*.

C′ killed cells of different orgaanisms revealed marked differences in their sensitivity to lysozyme (Fig. 3). For example, *S. typhi* Ty2 required 130 μg of the enzyme for conversion of 50% of its C′ killed cells to spheroplasts, whereas only 2.3 μg were needed with comparable cells of *S. typhi* 0901. Also of significance was the marked difference in the slope of the dose-response relationship observed with *S. typhi* Ty2 in comparison with the other organisms tested. Possibly the low slope with strain Ty2 may be related to its high level of Vi antigen which results in the inaccessibility of its lysozyme substrate either as a result of physical or chemical forces. Other possible factors contributing to differences in lysozyme sensitivity of C′ killed cells include quantitative as well as qualitative differences in the cell wall glycosaminopeptides that are the substrates of lysozyme. These compounds vary extensively in their detailed structures, for example, in the extent of peptide crosslinkage, degree of substitution with O acetyl groups, and the proportion of unsubstituted muramic acid residues in the oligosaccharide chains(13). Data are not available relative to these differences in the organisms used in these studies so that their significance, if any, in contributing to the experimental results cannot be assessed at this time.

Although the immune bactericidal reaction and lysis of *V. cholerae* has been indistinguishable experimentally, the killing of *S. typhi* organisms has been attributed to C′ and their subsequent lysis by lysozyme. This distinction may result from differences in the anatomical structures in *S. typhi* attacked by C′ and by lysozyme. The cell membrane has been implicated as the site of damage by C′

in the killing of gram-negative bacteria. For example, when treated with fresh normal serum, as a source of antibody and C′, *Escherichia coli* suffered considerable loss of phosphorus labeled compounds(14). In addition, pits or holes in *E. coli* similar to those seen in red cells as a result of immune hemolysis have been observed as a result of the action of C′(15). Finally, magnesium ion, which is capable of stabilizing cell membranes, may reverse the bactericidal action of C′ under certain experimental conditions(16). These results strongly suggest that the cell membrane is the target for the bactericidal action of C′, but they do not indicate why C′ killed cells are susceptible to gross lysis and disintegration by lysozyme whereas viable cells are neither lysed nor killed by the enzyme.

The C′ attack upon a bacterial cell which results in loss of permeability control and its death conceivably results in the uptake of water, swelling of the cells, and consequent exposure of the cell's lysozyme substrate. There is general agreement that lysozyme degrades the cell wall's insoluble polymers which constitute its rigid layer. Subsequent attack by lysozyme results, therefore, in a loss of the cell's structural rigidity which does not occur as a result of C′ action alone except perhaps in the case of *Vibrio* species. The cell wall of *V. cholerae,* unlike the cell walls of the *Enterobacteriaceae,* may require an intact cell membrane to maintain its rigidity. Although data for *V. cholerae* are not available, the percentage of lipid in the cell wall of *Vibrio metchnikovi* is 11.2 and the percentage of polysaccharide is 12.3, while comparable percentages for *Salmonella gallinarum* are 22 and 28 respectively(17). It is likely, therefore, that the cell walls of *Vibrio* species are less rigid and more easily ruptured. Other possible alternatives, however unlikely, include the activation of a cell wall autolytic process by C′ or the presence of lytic enzymes in serum, other than lysozyme, that are not removable by absorption with bentonite.

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