Protoplast Formation as the Mechanism for Immune Lysis of *Vibrio cholerae.** (28459)

BOB A. FREEMAN, GRAZINA M. MUSTEIKIS and WILLIAM BURROWS
Department of Microbiology, University of Chicago, Chicago, Ill.

The cholera vibrio is one of the few bacteria that is lysed as well as killed in the presence of homologous antibody and complement. It has been found in this laboratory that the two processes are differentiable with respect to time and rate(1). Vibriocidal activity is initiated immediately at maximum exponential rate and is substantially complete in 40-60 minutes depending upon the concentration of the reactants. In contrast, lysis as indicated photometrically is apparent only after a period of 20-30 minutes, continues at an exponentially increasing rate until 50-70% is attained in 2-3 hours. These findings would indicate that the bacteria are killed prior to lysis, and the relationship of the two processes is consistent with the assumption that the vibriocidal reaction is a prelude to the vibriolytic reaction.

Although the bactericidal activity of antibody and complement has been known for many years, the mechanism of the killing effect is poorly understood. The hypothesis that key metabolic reactions are affected by inactivation of relevant enzymes in combination with antibody has been an attractive one. It is not supported by available evidence since it has not been possible to establish adverse effects of antibody on respiration, viability, and growth(2,3,4).

There is some evidence, however, that respiration, as assayed by oxygen consumption, is inhibited in the presence of antibody and complement. While Sevag and Miller(5) were unable to show an inhibitory effect on pneumococcus, possibly because of heavy encapsulation, they found that the oxygen uptake of typhoid bacilli, though initially stimulated, was markedly reduced at 60 minutes, the extent depending upon the substrate supplied, and the bacteria were lysed. Bactericidal activity was not specifically determined. Similarly, inhibition of oxygen consumption of coliform bacilli, reaching a maximum within 30 minutes after addition of complement to the sensitized bacteria, but no inhibition in the presence of antibody alone, has been reported by Vargues *et al.*(6,7). While the evidence is fragmentary, it would appear that antibody alone does not appreciably alter respiratory activity, but metabolic activity may be inhibited in the presence of antibody and complement whether or not lysis occurs.

It is not clear that such effects, when observed, are primary or secondary, *i.e.*, whether inhibition of essential metabolic reactions by inactivation of relevant enzymes or whether they are secondary to lethal effects of some other nature. The requirement for complement, and the analogy with immune cytolysis in general, suggest that some structural alteration in the bacterial cell may occur which has lethal consequences whether or not actual lysis occurs.

The present report is concerned with the effect of antibody and complement on the cholera vibrio to give rapid formation of spherical, osmotically fragile, protoplast-like cells coincident with vibriocidal action and prior to lysis.

Materials and methods. The strain of *Vibrio cholerae* used here was an Inaba serotype isolated from a fatal case of the disease and given the series number C-401; its history has been described(8). The cell suspensions were prepared in physiological saline, pH 8.0, from 16 hour cultures on thionin-glycerol agar, and the density adjusted photometrically to contain 1×10^9 cells/ml. A 1:5 dilution of Vacseal complement and a 1:5,000 dilution of a pooled hyperimmune serum prepared in the rabbit were the other two reagents.

The reaction was carried out at 37°C in volumes of 5 ml per tube in a series of tubes, one being used for each sampling. Each contained 1 ml of antiserum, 1 ml of comple-

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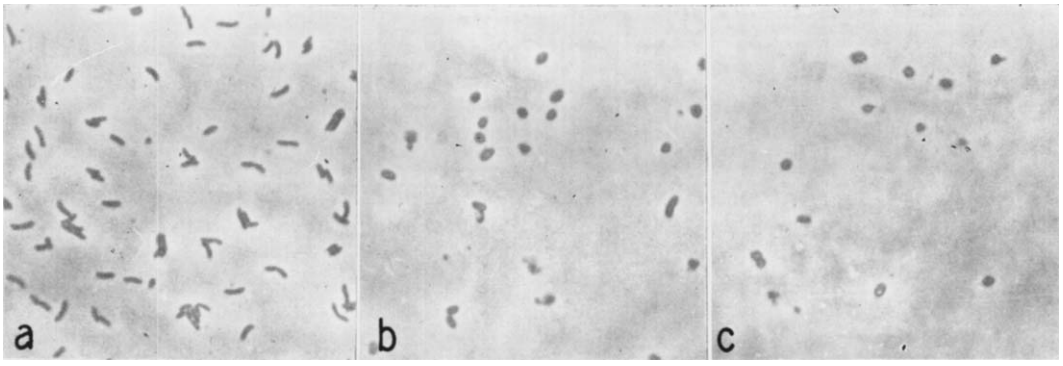


FIG. 1. Normal vibrio (a) and antibody-complement induced protoplast-like forms (b, c) of the cholera vibrio. (b) Early morphologic types of protoplast-like forms; note the swollen vibrio and forms with tail-like appendages. (c) Later morphologic types showing empty and disintegrating forms. Living preparations under medium dark phase contrast. X 2000.

ment, 1 ml of bacterial suspension, and 2 ml of saline, giving final dilutions of 1:25,000 of antiserum and 1:25 of complement, and containing 200 $\times$ 10^6 vibrios/ml. The velocity of the vibriocidal reaction is a function of the concentrations of these reactants(1); a detailed kinetic study to be described elsewhere provided the basis of the suitability of the foregoing concentrations for present purposes.

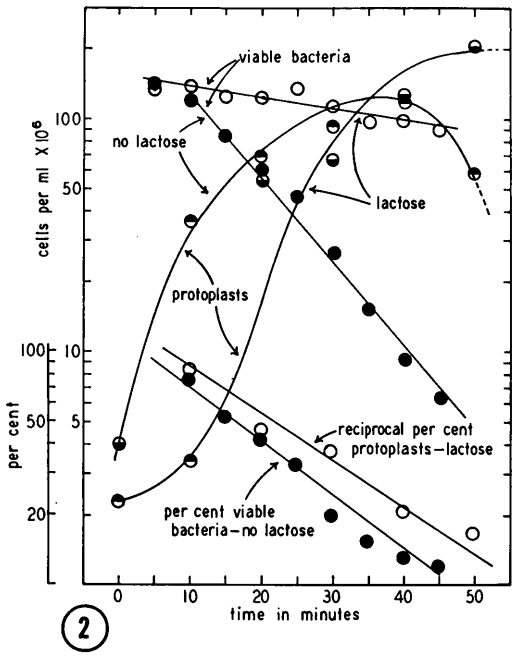
Numbers of viable bacteria were assayed on the basis of the linear relation between numbers of viable bacteria in the inoculum and the length of the lag period in culture as described by Treffers *et al.*(9,10). As carried out here, 1 ml portions of the reaction mixture were inoculated into 5 ml of prewarmed trypticase soy broth, pH 8.0, and incubated as maximally aerated cultures. Growth was measured photometrically at intervals and the midpoint of the logarithmic growth phase (300 on the Klett-Summerson scale with 16 mm culture tubes) was arrived at by interpolation. The number of viable cells is given by a previously determined linear relation of numbers and time required to reach the growth endpoint.

Protoplasts were observed in wet mounts under oil immersion by medium dark phase contrast microscopy, using a green filter. Protoplast and bacterial counts were made on formalin-fixed preparations in a Petroff-Hausser chamber.

Results. The conversion of vibrios to spherical, protoplast-like forms in the presence of antibody and complement was observed qualitatively in wet mounts of material taken at 5-10 minute intervals during incubation. In the initial stages, the vibrios became somewhat swollen and nonmotile prior to rounding up, and in some cases the rounding-up tended to occur terminally to give intermediate forms with a short tail-like appendage. The fully spherical forms were at first homogeneous under medium dark phase contrast, but later showed internal irregularities, and in the late stages of incubation, shrunken empty forms appearing as dark rings were found together with debris presumably representing lysed forms. These protoplast-like forms are illustrated in Fig. 1.

Total counts of vibrios and protoplasts in formalin-fixed preparations in the Petroff-Hausser chamber showed that about half the vibrios were converted to the protoplast-like form in the presence of 1:25,000 antiserum and 1:25 complement in about 30 minutes, and the proportion approached 90% in 50 minutes. In the absence of osmotic protection, total cell count began to decline very early, to 64% in 30 minutes, to 24% in 50 minutes, and to 1% in 120 minutes. This decrease in total numbers could be accounted for substantially entirely by conversion of the vibrios to protoplasts and subsequent disappearance of the protoplasts.

Protoplasts were not produced, nor were vibrios killed, when the complement was heat-inactivated or when distilled water supplemented with Ca^{++} and Mg^{++} was substituted



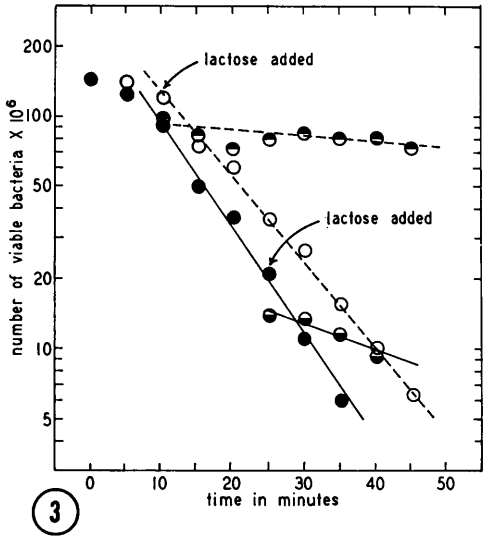


FIG. 2. Upper portion: Correlation of total protoplast and viable bacterial counts during immune vibriocidal reaction in presence and absence of 0.5 M lactose. Lower portion: Comparison of rates of protoplast formation in presence of lactose, and vibriocidal reaction in absence of lactose.

FIG. 3. Inhibition of immune vibriocidal reaction by addition of lactose to 0.5 M concentration. In the experiment shown by dash lines, lactose was added to one portion of the reaction mixture after 10 minutes incubation. The protective effect is shown by sharply reduced rate of killing in presence of lactose in contrast with continued killing effect in its absence. In the experiment shown by solid lines, addition of lactose after 25 minutes of incubation was similarly protective.

for 0.85% NaCl. The reaction did not require additional Ca^{++} and/or Mg^{++} in the saline as has been reported(11).

While sucrose provided osmotic protection and preserved the protoplasts, the bacteria were killed by the acidity resulting from rapid fermentation of the sugar. Of various other substances tested, lactose in 0.5 M concentration provided osmotic protection although it has not been found satisfactory for Shigella protoplasts(12). There was a slight lag in protoplast formation in lactose-saline as compared with saline alone when observed microscopically.

Lactose also protected against the vibriocidal activity of the antibody-complement system. The protection was evident only when lactose was present in the reaction mixture, and the sugar had little or no protective effect when included in the subculture medium. Some degree of protection was apparent even at relatively high antibody concentrations, but the antiserum could be diluted until the lactose gave substantially complete protection while in its absence the vibriocidal rate was such that half the bacteria were killed in 20-30 minutes. The dilution of the antiserum used was 1:25,000. The concentrations of complement and bacteria also affect the rate of the vibriocidal reaction(1), as will be described elsewhere in detail, but have not been studied here in relation to protoplast formation.

A representative experiment in which total counts of vibrios and protoplasts were carried out in conjunction with assays of viable numbers in presence and absence of lactose in the reaction mixture is shown in Fig. 2. It is evident from these data that 0.5 M lactose suffices to protect against the bactericidal activity. Further, the association of the killing effect with the relatively rapid formation of protoplasts is apparent in the total counts, together with the persistence of protoplasts in the presence of lactose and disappearance, beginning at 30-40 minutes, in its absence.

Comparison of the survival and protoplast data in the absence of lactose shows that the rate of protoplast formation is somewhat

more rapid than that of the vibriocidal reaction. The protoplasts, therefore, appear to remain viable for a time as assayed by the subculture method. In the lower part of Fig. 2 the rate of killing as percent of viable bacteria remaining in the absence of lactose, and the reciprocal of the percent of total cells appearing as protoplasts when protected by lactose are shown. It is relevant here that the rates are substantially the same; the displacement of the rate of protoplast formation to the right is a consequence of the lag in protoplast formation in the presence of lactose as shown in the same figure.

Osmotic fragility of these protoplast-like forms is implied by their persistence in lactose and destruction in saline as observed directly in total counts. That it is intimately related to the vibriocidal reaction may be shown by addition of lactose to the reaction mixture in saline, or by dilution of the lactose-containing reaction mixture in saline, and assay of survivors. A representative experiment of the former kind is shown in Fig. 3. Here lactose was added after 10 minutes incubation in one series, and after 25 minutes incubation in the other. It is evident that the vibriocidal reaction is markedly inhibited in that portion of the mixture to which lactose was added, even after more than 80% of the bacteria had been killed at 25 minutes. In the converse kind of experiment, *i.e.*, in which the lactose-containing mixture was diluted 1:10 during the course of the reaction, the vibriocidal effect became immediately apparent on dilution even though dilution was delayed as late as 25 minutes of incubation.

Discussion. The formation of protoplast-like cells of gram-negative bacilli in the presence of fresh normal serum containing complement activity, or supplemented with complement after inactivation or removal of complement by antigen-antibody precipitates, has been described by a number of workers. Michael and Braun(12) prepared protoplast-like forms, designated spheroplasts, of *Shigella dysenteriae* by exposure to fresh normal serum in low dilution (1:2.5 to 1:6) in the presence of sucrose. When the serum was absorbed with dysentery bacilli these forms were no longer produced. Since absorption with *Escherichia coli* did not have this effect, these authors suggested that small amounts of antibody present in normal serum were significant. The role of antibody was pointed up in the studies of Muschel *et al.*(11) in which the conversion of typhoid bacilli to protoplasts required specific antibody and complement and, in the case of smooth forms, lysozyme. These workers expressed the view that the formation of protoplasts is a 2-stage process in which the bactericidal action of antibody prepares the cells for conversion to protoplasts by lysozyme. They inferred from this that the cells are killed prior to depolymerization of cell wall substance by lysozyme to give a protoplast-like form, and, in fact, ultraviolet-inactivated cells could also be converted to protoplasts by lysozyme. Wardlaw's(13) observations on the bactericidal activity of normal human serum, used in low dilutions of 1:10 to 1:20, on coliform bacilli were also taken to indicate a primary role of lysozyme. Absorption of serum resulted in removal of lysozyme concurrently with reduction in bactericidal activity, and the activity was said to be restored by addition of lysozyme; antibody was considered to play no part in the reaction. But the role of antibody was further substantiated by Muschel(14) who showed that lysozyme was ineffective in the presence of serum from which antibody had been completely exhausted, but effective when small amounts of antibody were added to the system.

Certain of the foregoing observations have been taken to suggest that the activity of lysozyme is an essential element in the immune bactericidal reaction, but this view no longer seems tenable. It does not follow that when the bactericidal activity is reduced by absorption with the bacterial antigen and subsequently enhanced by addition of lysozyme, that the lysozyme activity is "restored." It is not only equally possible that the increased activity is supplemental rather than restorative, but it has been shown(14), as indicated above, that lysozyme is inactive in the presence of exhaustively absorbed antiserum and functional only when the bacterial substrate has been sensitized by anti-

body and complement. Further, the irrelevance of lysozyme to the immune vibriocidal reaction is clearly shown in the studies described here in which the reaction occurred in the absence of detectable amounts of lysozyme. Two lots of complement used were examined for lysozyme content by the conventional titration with *Micrococcus lysodeikticus* and found to contain <0.25 μg/ml; in a 1:25 dilution of complement in the system used here the lysozyme content of the reaction mixture was <0.01 μg/ml.

The immune bactericidal reaction may perhaps be supplemented or enhanced by the activity of lysozyme when sufficiently large amounts of lysozyme-containing serum are present *in vitro*. For example, certain of Wardlaw's(13) sera contained as much as 5.4 μg/ml of lysozyme and were used in dilutions of 1:10, and Muschel *et al.*(11) used very large amounts of complement as a 1:1 dilution of guinea pig serum. Similar enhancement might also occur *in vivo*. Nevertheless, the nature of the immune bactericidal reaction *per se* seems not to involve the activity of lysozyme.

It is evident from the observations reported here that the cholera vibrio is rapidly converted to a protoplast-like, osmotically fragile form in the presence of specific antibody and complement, the former in high dilution and the latter in conventional amounts. The intimate relation between osmotic fragility and the immune vibriocidal reaction is clearly indicated by the correspondence between protoplast formation and death of the microorganisms, and by the prevention of the vibriocidal reaction by the osmotic protection conferred by 0.5 M lactose incorporated in the reaction mixture, interruption of killing when the osmotic protection is added during the course of the reaction, and initiation of killing when the osmotic protection is removed by dilution. It is suggested, therefore, that the primary effect of antibody and complement on the cholera vibrio is a breakdown of the cell structure to give an osmotically fragile but viable form. There is no evidence that the cell wall is completely removed. In fact, the persistence of forms with a tail-like appendage, perhaps representing residual cell wall material, suggests that it is not. Such forms should be designated protoplast-like, spheroplasts, etc. rather than protoplasts(15), but in the present context the essential point is the osmotic fragility rather than nomenclature.

In the absence of osmotic protection these forms may remain viable for a short time, but very soon they are incapable of reproducing on subculture, possibly as a consequence of leakage of cell contents or other effects on the osmotic regulatory mechanisms. Eventually these forms lyse leaving only debris, or deteriorate to a shrunken empty particle. Thus the immune vibriocidal and vibriolytic reactions are secondary rather than primary consequences of the antigen-antibody-complement union. Such a mechanism of the immune bactericidal reaction may be generalized to include bacteria which do not undergo obvious immune lysis if it be assumed that the effect on osmotic regulatory mechanisms need not have a morphological reflection.

Summary. It is shown that cholera vibrio is converted to a protoplast-like, osmotically fragile form in the presence of specific antibody and complement. When osmotic protection is provided by 0.5 M lactose in the reaction mixture, the bacteria are protected from the vibriocidal effect of antibody and complement. Protection is conferred during the course of the reaction as indicated by cessation of killing when lactose is added; and disappears with onset of the vibriocidal reaction when osmotic protection is removed by dilution of the lactose-containing reaction mixture. In view of the apparent intimate relationship between the vibriocidal reaction and osmotic fragility, and the substantial identity of the rates of killing and protoplast formation, it is suggested that the primary consequence of the antigen-antibody-complement union is the formation of osmotically fragile, protoplast-like forms to give, in the absence of osmotic protection, the vibriocidal and vibriolytic reactions as secondary phenomena.

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Free Amino Acids in Milk.* (28460)

MARVIN D. ARMSTRONG and KERIN N. YATES
Fels Research Institute, Yellow Springs, Ohio

Although a large body of data is available on the total amino acid content of milk, there is little information about the free amino acids. Investigations on amino acid excretion of infants showed that at least one, later shown to be homocitrulline(1,2), must originate in milk, and it seemed possible that other unusual amino acids might be present in small amounts, either free or in proteins. It was, therefore, of interest to examine the free amino acids in milk for 2 reasons: first, to see if there are any new or unique components and, second, because some information might be gained about the intracellular environment of milk-secreting cells. This note reports values for the concentration of free amino acids in milk from 3 healthy, nursing mothers under fasting conditions, with concomitant measurements of the amino acids in serum and urine collected at the same time. Values obtained with cow's milk are also included.

Experimental procedures. Three mothers were used as subjects; samples were collected from subject A 4 weeks, and from K and S 3 months after delivery. They had not eaten for 14 hours and their infants had not nursed for 12 hours before the samples were taken. Milk was expressed manually and approximately 30 ml was obtained from each breast. The milk was centrifuged immediately, the lipid layer was removed and discarded, and a protein-free filtrate was prepared with an ARAFLO (Applied Research Corporation, N. Y.) cell using nitrogen pressure. One drop of 3 *N* HCl was added to a 10 ml sample of the filtrate which was then concentrated *in vacuo* at room temperature to a volume of about 1 ml. This was made up to a volume of exactly 5.0 ml by addition of pH 2.2 citrate buffer, 0.2 *N* in Na^+ and stored at −15° until the analyses were made. Blood was drawn from the antecubital vein, allowed to clot at room temperature for 7 hours, and a serum filtrate was prepared for analysis in the manner described for milk. The subjects voided when they arrived at the laboratory, and a urine sample was collected after the blood was taken. The creatinine content was determined and the urine was stored at −15° until the analyses were made.

Milk was collected directly from Jersey cows, with no attempt to regulate time of collection, centrifuged just after collection to separate lipids, and filtrates were prepared immediately. In addition to pressure filtration, sulfosalicylic acid filtrates were prepared by a modification of the method of Hamilton (3).

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