Normal Serum Cytotoxicity for P³²-Labeled Smooth *Enterobacteriaceae*

I. Loss of Label, Death, and Ultrastructural Damage

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ABSTRACT

SPITZNAGEL, JOHN K. (University of North Carolina, Chapel Hill), AND LAWRENCE A. WILSON. Normal serum cytotoxicity for P³²-labeled smooth *Enterobacteriaceae*. I. Loss of label, death, and ultrastructural damage. *J. Bacteriol.* 91:393-400. 1966.—Metabolically labeled smooth *Escherichia coli* lost between 10 and 90% of P³², compared with control suspensions, when suspended for 60 min in normal serum at 37 C. Similar results were obtained with several other genera of *Enterobacteriaceae*. The structural nature of the cell injury in *E. coli* was shown by electron microscopic examination of ultrathin sections. Complex injury which included all peripheral cell structures occurred and various degrees of cytoplasmic loss resulted. Injured bacteria retained their essential shape and did not collapse, evidently because sufficient rigid cell wall remained to prevent this. The cell damage was more like that reported with ethylenediaminetetraacetic acid lysozyme than that reported with growth in penicillin. Damage sufficient to cause rupture of peripheral structures was unique in that it involved only localized areas of cell wall and left large sections relatively intact. The loss of P³² in the fresh normal serum was closely related to bacterial viability loss.

The expression of toxic, antigenic, and other biological properties of gram-negative bacteria may depend on the physical and chemical form of their in vivo degradation products. Studies of serum antibacterial action which utilize the usual measurements of bactericidal, bacteriolytic, or opsonic effects yield little information concerning end products. Comprehension of the contribution of normal serum factors to the degradative process requires precise knowledge of the molecular and structural targets affected as well as the pathways and rates of degradation. The antibacterial effects of normal serum for *Enterobacteriaceae* are extensively documented [reviewed by Skarnes and Watson (16)]. More recent studies include those by Amano et al. (1), Inoue et al. (3), Muschel, Carey, and Baron (9), Michael, Whitby, and Landy (8), Osawa and Muschel (11), and Wardlaw (20). Most of these studies are based on classical measurements of bactericidal or bacteriolytic effects. Only the studies of Amano et al. (1) and of Rowley (14) suggest the possible

effects of normal or hyperimmune serum on microbial constituents such as macromolecular phosphorous compounds. Structural damage to *Enterobacteriaceae* has been reported primarily in terms of microscopically visible lysis or spheroplast formation in rough strains (1, 3, 9, 20).

Bacterial cells labeled metabolically with radioactive nuclides such as P³² provide the advantages of modern tracer techniques for studies of in vivo degradation of bacteria. Isotopic labeling techniques have been used in several recently published studies of opsonization, phagocytosis and intracellular destruction of bacteria [reviewed by Suter and Ramseir (18)]. Interpretation of results obtained with labeled bacteria may be complicated by various factors, including isotope exchange and the effects of ill-defined but unavoidable system components such as normal adult serum. It is unfortunate that the authors cited provide little indication of the possible influence of these effects on the outcome of their studies.

Because normal serum may participate in in vivo microbial degradation, even intracellularly

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(4), and because serum is so often a part of in vivo microbial environment, it is important to know its effect on labeled bacteria. As will be seen, the use of labeled bacteria also gives a sensitive method for studying the antibacterial action of serum as such. The subject of this and two other papers is the effect of normal adult mammalian sera on the composition, viability, and ultrastructure of smooth *Enterobacteriaceae*, especially *E. coli*. This paper shows that labeled smooth, as well as rough, strains lose large amounts of P^{32} in fresh normal adult mammalian serum. In the smooth *E. coli*, loss of P^{32} is related to severe ultrastructural damage and death.

MATERIALS AND METHODS

Cultures. *Escherichia coli* O117:H27 (17) and O26:B6 (kindly supplied by W. H. Ewing of the Communicable Disease Center, Atlanta, Ga.), *E. coli* K-12, and *Klebsiella pneumoniae* (Julianelle No. 4208) were maintained on nutrient agar slants. Periodic immunological and biological checks assured the properties of these organisms. *Proteus morganii*, *Serratia marcescens*, *Shigella paradysenteriae*, and *Salmonella typhosa* were from the culture collection of the Department of Bacteriology and Immunology.

Media. Nutrient agar and antibiotic assay broth (Difco) were used for maintenance and growth of stock cultures. A complex synthetic medium (6) composed of amino acids, vitamins, and salts with sufficient phosphate salts (0.4 mmoles of phosphate per liter) to permit three to four divisions of *E. coli* in 4 hr was used for labeling cells with P^{32} and to produce bacterial cell walls. Amino acids were supplied at 10 times the concentration recommended for tissue culture. No glucose was used, nor was any additional buffer used. The hydrogen ion concentration began at 7.0 and rose to 8.0 during labeling. Gey's balanced salt solution with glucose (200 mg per 100 ml) for equilibration with air was prepared according to Paul (12). This choice of media and saline solutions resulted from our intent to use the labeled organisms in phagocytic experiments in which these media were to be used.

P^{32} labeling. A culture of the organism to be labeled was grown for 4 hr in a shaking water bath, washed three times, and brought to original volume in sterile water. A 40-ml amount of labeling medium containing 10 μ C of P^{32} as orthophosphate was inoculated with 0.5 ml of the washed culture and then was incubated for 4 hr with shaking at 37 C. The bacterial cells (usually 0.75 mg/ml of *E. coli*) were washed three times in 0.1 M phosphate buffer (pH 7), suspended to volume in Gey's solution, and stored at 4 C.

Cell walls. Cell walls were obtained by the procedure of Roberson and Schwab (13) from *E. coli* O117:H27 grown in 1-liter batches in the labeling medium.

Animal serum. Aseptically collected blood of apparently healthy animals was allowed to clot at room temperature. The serum was collected and stored at -20 C.

Radioisotope techniques. Phosphorus (P^{32}) obtained from Oak Ridge National Laboratories, Oak Ridge, Tenn., was hydrolyzed in acid, and the purity of the orthophosphate was ascertained chromatographically. Radioactivity was measured with a PC3 windowless proportional counter (Nuclear Measurements Corp., Indianapolis, Ind.), or a Scintillation Spectrometer (Packard Instrument Corp., Downers Grove, Ill.). In all cases, total counts were large enough to give no more than a 6% error at the 95% confidence level, and in most cases total counts sufficed to give a 3% error at the 95% level.

Release of P^{32} from labeled bacteria. Final reaction volume was 2.1 ml. Labeled bacteria, 0.5 mg (dry weight) per ml or less, were suspended in Gey's solution containing the desired concentrations of other reactants. Serum concentrations were 0.5 ml or less of whole serum per ml of final reaction volume, as the protocols demanded.

Total P^{32} activity of the bacterial suspension was determined by use of a sample of the inoculum equal to the final bacterial inoculum per tube. Reaction tubes, maintained at 4 C during preparation, were centrifuged at $6,000 \times g$ for 10 min at 4 C, and 100 μ l of the clear supernatant fluid was removed for P^{32} estimation (zero-time P^{32} concentration). After incubation at 37 C for 60 min, the tubes were again centrifuged at $6,000 \times g$ for 10 min at 4 C. Again, 100 μ l of the clear supernatant fluid was removed and the P^{32} content was measured (60-min P^{32} concentration).

Calculation of P^{32} loss per hour. After making appropriate corrections for background counts and efficiency of the instruments, volume corrections were applied and the percentage of P^{32} released per hour was calculated as

$$\frac{P_f^{32} - P_i^{32}}{P_t^{32} - P_i^{32}} \times 100 = \text{per cent } P^{32} \text{ lost per hour}$$

where P_f^{32} was the final concentration of P^{32} per milliliter of supernatant fluid, P_i^{32} was the initial P^{32} concentration in the supernatant fluid, and P_t^{32} was the total concentration of P^{32} in the initial bacterial suspension.

Cell counting and sizing. Cell counts and cell sizing were done according to Mattern et al. (7). An electronic particle counter (Coulter Type "A", Coulter Electronics, Miami, Fla.) equipped with a 30- μ counting aperture was used. T division = 0.0193 μ^3 .

Electron microscopy. Electron microscopy was done by use of an Akashi instrument (Bendix Corp., Cleveland, Ohio). Ultrathin sections were prepared by a modification of Kellenberger's technique with Maraglas (Polysciences Inc., Rydel, Pa.) used as an imbedding medium. Sequential straining of sections with uranyl acetate and lead hydroxide increased contrast.

Bactericidal assay. Bactericidal assay was done according to Muschel's (10) technique except that media used were those specified above. Labeled viable bacteria (0.005 mg/ml) were used, and both P^{32} loss and bacterial killing were measured simultaneously from the same reaction tubes. Gey's solution may be

toxic to *E. coli*. Under the conditions of bactericidal assay used here, this was not so, possibly because of carry-over of nutrients in the bacterial inoculum.

RESULTS

Effects of fresh frozen serum on P^{32} of labeled Enterobacteriaceae. When P^{32} -labeled *E. coli* O117:H27 cells were suspended for 1 hr at 37 C in medium containing 50% fresh frozen guinea pig serum (see Table 1) 40% of the P^{32} label entered the suspending medium. The released label

TABLE 1. Leakage of metabolically incorporated P^{32} from *Escherichia coli* O117:H27 in the presence of various fresh animal sera^a

Serum source	Total sedimentable P^{32} at zero-time	Nonsedimentable at $6,000 \times g$ for 10 min		Bacterial P^{32} lost into suspending medium/hr
		Zero-time	After 60 min at 37 C	
Guinea pig...	163,400 ^b	376	65,848	40 ^c
Dog.....	157,830	496	52,960	33
Rat.....	160,470	1,280	56,368	35
Rabbit.....	179,150	808	43,960	24
Blue crab.....	149,710	232	26,202	17
Gey's solution.....	160,690	176	23,616	14
Man ^d	40,467	1,810	25,320	58
Gey's solution ^d	40,367	1,910	3,790	5

^a Fifty per cent serum in Gey's solution.

^b Counts per minute per milliliter.

^c Percentage of total P^{32} in original suspension bacteria.

^d Separate experiment.

TABLE 2. Leakage of metabolically incorporated P^{32} from Enterobacteriaceae suspended in fresh guinea pig serum

Species	Suspending medium	
	Gey's solution	50% serum-Gey's solution
<i>Escherichia coli</i> O117:H27....	5*	31
<i>E. coli</i> K-12.....	1	68
<i>Salmonella typhosa</i>	3	29
<i>S. paratyphosa</i>	9	5
<i>Klebsiella pneumoniae</i>	1	15
<i>Proteus morganii</i>	2	47
<i>Serratia marcescens</i>	10	40

* Per cent P^{32} lost into suspending medium per hour at 37 C.

FIG. 1. Size distribution curve of *Escherichia coli* suspended in Gey's solution (solid line) or in 50% fresh guinea pig serum-Gey's solution (broken line) and incubated at 37 C for 1 hr. Calibration with standard latex particles showed $T \times 1 = 0.0193 \mu^2$.

TABLE 3. Comparative sedimentation* of particle-bound P^{32} released from *Escherichia coli* O117:H27

Gravitational field	Time	Suspending medium	
		Gey's solution	Serum-Gey's solution
	min		
$6,000 \times g$	15	15,376†	109,690
$35,000 \times g$	30	—	104,120
$80,000 \times g$	30	15,816	85,668
$100,000 \times g$	30	8,228	72,656

* Sedimentation was done in the medium the organisms were incubated in. The higher specific gravity and viscosity of the 50% serum medium probably caused the particles contained in it to sediment less rapidly than those in the plain Gey's solution.

† Counts per minute per milliliter of supernatant fluid.

could not be sedimented at $6,000 \times g$ for 10 min, the force used to sediment the remaining bacterial particles. More P^{32} was released in serum than in serum-free Gey's balanced salt solution. All of the sera of several species, including man, caused release of P^{32} that exceeded that observed in Gey's solution (Table 1). Invertebrate serum (blue crab) had no effect on the P^{32} label.

Enterobacteriaceae labeled with P^{32} . *E. coli* K-12 (an essentially rough organism), a smooth *Salmonella typhosa*; a smooth *K. pneumoniae*, *Serratia marcescens*; *P. morganii*, and *Shigella paratyphosa* were tested in the same way (Table 2). All except one of the serum-suspended cultures

FIG. 2A. Control *Escherichia coli* O117:H27 cells after 1 hr at 37 C in Gey's solution. Note wrinkled appearance of cell periphery. N, nucleoplasm; C, cytoplasm; CW, cell wall; CM, cytoplasmic membrane.

FIG. 2B. *Escherichia coli* O117:H27 after 1 hr at 37 C in fresh guinea pig serum. Cytoplasm and nucleoplasm are gone; the cytoplasmic membranes (CM) are clearly visible in some parts of cells and injured or absent in others. There is a tendency for cell walls (CW) to be separated from cytoplasmic membranes. Rupture of all peripheral membranes (X) is seen in some places. At WX, the otherwise intensely stained wall structure is pale and thinned between the arrows.

FIG. 2C. *Escherichia coli* O117:H27 as in Fig. 2B. This micrograph shows shapes of cells, damaged CM, and damaged CW. A relatively unscathed (NR) organism can be seen, but the peripheral layers are smooth and the wall is injured (X). Space between CM and CW is exaggerated.

lost from 6 to 70 times more P^{32} than did organisms suspended in Gey's solution. The greatest loss of P^{32} occurred from the rough *E. coli* K-12. The P^{32} label of *S. paratyphenteriae* was not affected by serum-Gey's solution differently from saline.

Effects of guinea pig serum on size distribution of E. coli. *E. coli* cells [0.53 mg (dry weight) per ml] labeled with P^{32} were suspended in 50% fresh normal guinea pig serum and an equivalent suspension was made in 100% Gey's solution. After 1 hr at 37 C, 45% of the P^{32} had been re-

FIG. 3A. *Escherichia coli* O117:H27 as in Fig. 2A but enlarged 84,000 times. N is the nucleoplasm surrounded by granular cytoplasm (C), which in turn is surrounded by cytoplasmic membrane (CM). Cell wall is seen as a wrinkled outer layer.

FIG. 3B. *Escherichia coli* O117:H27 as in Fig. 2B. The figure is enlarged 84,000 times to show an injured cell with much reduced granular cytoplasm (C) and redistributed nucleoplasm (N). An outer peripheral membrane (PM) with three layers is seen. In addition, CW and CM are present. At X, the CM appears damaged with pufflike extrusions of cytoplasm seen in the space between CM and CW.

FIG. 3C. *Escherichia coli* O117:H27 as in Fig. 2B. Here, extensive fractures (X) have broken the CW. The CM is very much altered or absent. The inward curling of the CW is seen.

FIG. 3D. *Escherichia coli* O117:H27 as in Fig. 2B. A detail of cell-wall injury (X) is seen. The triple layering of CM and CW are well resolved.

leased from the bacteria in serum-Gey's solution. The number and size distribution of the cells in each suspension were estimated (Fig. 1). Size distribution for 60% of the cells incubated in

serum-Gey's solution was about a mean T of 42, and for 38% was about a mean T of 28. Size distribution for 73% of the cells incubated in Gey's solution was about a mean T of 50 and

FIG. 4. Dose-effect curve of bactericidal serum action on P^{32} -labeled *Escherichia coli* O117:H27 (●) compared with dose-effect curve for loss of P^{32} from the same cells (■) into the suspending medium.

for 25% was about a mean T of 28. The total number of bacteria per milliliter remained the same in both the serum-Gey's solution and the Gey's solution suspension.

The cell-free supernatant fluids remaining after centrifugation at $6,000 \times g$ were centrifuged with increasing force for 30-min periods. Table 3 shows that the P^{32} labeled particles released in serum-Gey's solution were sedimented more readily and were, therefore, probably larger than those released in plain Gey's solution.

Effects of guinea pig serum on morphology of *E. coli*. Phase-contrast microscopy revealed little change in the shape of *E. coli* O117:H27 suspended in 50% serum-Gey's solution. The bacteria were immobilized and agglutinated. *E. coli* K-12 and a strain of *E. coli* O26:B6 which had become slightly rough formed "serum spheroplasts" (1), and were agglutinated as well as immobilized.

Ultrastructure of peripheral membranes and cytoplasm of bacteria (Fig. 2A and 3A) incubated in Gey's solution resembled that reported for *E. coli* by Kellenberger (5). The reason for the appearance of the nucleoplasm in our preparations was not clear, but it may have been due to the media in which the experiments were done. Serum-treated bacteria that had lost 60% of their P^{32} showed retention of general bacterial shape and size (Fig. 2B, 2C, and 3B). However, damage to the cells was complex and extensive. Both moderately swollen and shrunken forms were present.

All but a few of the bacteria had lost most of their cytoplasm and internal structure (Fig. 2B and 2C). The bacterial remains included an inner cytoplasmic membrane (60 to 125 Å thick) (5) that in places was seen to have three layers (Fig.

3D). A triple-layered cell wall 60 to 125 Å thick which appeared relatively rigid was present in injured bacteria (5) (Fig. 3B, C, and D). A third membrane, also occasionally seen to have three layers (Fig. 3B), was found at the periphery of some organisms. It was sharply separated from the cell wall by an electron-transparent layer 20 to 24 Å thick. Cytoplasmic membranes were separated from cell walls (Fig. 2B, 2C, and 3B) and appeared most damaged with numerous small interruptions (Fig. 2B, 2C, 3B, and 3C). The cytoplasm appeared to extend through these holes between cytoplasmic membranes and cell walls (Fig. 2B and 3B). Cell-wall defects were less frequent but larger. Small bits of rolled up wall consisting of the outer two layers were numerous (Fig. 2B, 2C, and 3C). The outermost layer often appeared to be a mixture which included leaked cytoplasmic particles. All fresh serum-suspended cells were damaged, but in differing degrees.

Comparison of P^{32} -releasing and bactericidal actions of guinea pig serum. A bactericidal assay system that permitted simultaneous determination of 50% end points (10) for bactericidal action (LD_{50} *E. coli*) and P^{32} -releasing ($P^{32}L_{50}$) action of normal serum was used. The percentage of total bacterial P^{32} released (ordinate) or the percentage of bacteria surviving was plotted against the serum concentration per milliliter of final reaction mixture (abscissa) by use of logarithmic probability paper. A straight line resulted. Fifty per cent points were determined by inspection. The $P^{32}L_{50}$ of fresh guinea pig serum (Fig. 4) was 0.09 ml of serum per ml of a suspension of 10^7 *E. coli* per milliliter [0.005 mg (dry weight) per ml]. The LD_{50} *E. coli* of the serum was slightly less. The difference was within the 10% error inherent in the method for LD_{50} *E. coli* (10). The slopes of both dose-effect lines appeared to be similar, but certainty concerning this point requires greater refinement of technique.

DISCUSSION

Blood serum from "normal" adult animals caused major cellular loss of phosphorus compounds from smooth as well as rough phase *Enterobacteriaceae*. The identity and form of the compounds released has been developed in another paper in this series (17a). Judged by comparative sedimentation results, some of the P^{32} -labeled fragments, possibly ribonucleic acid or deoxyribonucleic acid, were still highly polymerized. Fragment size suggested that severe damage to bacterial permeability had occurred. Under specified conditions in whole fresh or fresh frozen guinea pig serum, phosphorus loss

and cell death were found to be closely related. The cytotoxic serum components that caused these effects included "normal" antibody, complement, and lysozyme (17b).

Serum damage to smooth *E. coli* appeared to involve both cytoplasmic membranes and cell walls. Loss of cytoplasm and nucleoplasm may have been secondary to damage to peripheral structures. The sequence of structural damage was not revealed, nor was the sequence or loci of action of the various serum components. Structurally, cells were damaged in differing degrees. Damage was not "all or nothing."

A type of rough *E. coli* spheroplast caused by depolymerization of cell-wall mucopeptide is typically produced by ethylenediaminetetraacetic acid (EDTA)-lysozyme. Spheroplasts caused by inhibition of mucopeptide synthesis with mucopeptide dilution find a prototype in rough *E. coli* grown in the presence of penicillin (15). Hofschneider (2) published ultrastructural studies of both spheroplast types. Ultrastructural changes in peripheral structures of normal serum-treated smooth *E. coli* most resembled those of rough *E. coli* treated with EDTA-lysozyme, especially with respect to the separation of the cell-wall layer (2).

Smooth *E. coli* treated with fresh serum in non-hypertonic media and seen by light microscopy did not become rounded and burst as do rough *E. coli* (1, 3). Smooth *E. coli* differed further in that their cell walls retained much more rigidity and structural complexity. When damaged by normal serum, smooth *E. coli* behaved as if sharply localized depolymerization of cell walls had facilitated ultrastructural damage with rupture resulting at points of injury. Assuming that lysozyme caused the depolymerization, this could mean that smooth *E. coli* have relatively fewer lysozyme-susceptible sites than do rough *E. coli*. Alternatively, the normal antibody in serum possesses specificities for fewer sites on smooth *E. coli* and is thus able to prepare fewer sites for lysozyme.

The opsonizing action of normal serum has usually been considered an action of normal antibody and complement at the bacterial surface and a step preparatory to phagocytosis (4, 19). Our results showed that opsonizing concentrations of normal serum caused severe injury to bacterial surfaces, destroyed permeability barriers, and initiated degradation of smooth *E. coli*. These serum actions may influence intraphagocytic microbial death and destruction. Indeed, Jenkin (4) has shown that serum opsonization of salmonellae accelerates their death in mouse peri-

toneal macrophages independently of effects on phagocytic rate.

Our results confirm and extend the work of other investigators on the antibacterial action of normal sera for *Enterobacteriaceae*. The model described has practical utility in the study of action of normal or hyperimmune serum components on *Enterobacteriaceae*, rough or smooth. It should also be useful in studying relationships between peripheral cell structure and molecular and antigenic composition.

Inoue et al. (3) and Muschel et al. (9) presented evidence based on light microscopy that bactericidal effects of antibody-complement for gram-negative bacteria may occur independently of spheroplast formation. Our results suggest that spheroplast formation may only be a severe example of cell-wall damage which, should it occur in only one locus, could still be lethal to the bacterial cell in hypotonic media.

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