INFECTION AND IMMUNITY, Apr. 1974, p. 663-668

Vol. 9, No. 4
Printed in U.S.A.

Persistence of Regulation of Macromolecular Synthesis by *Escherichia coli* During Killing by Disrupted Rabbit Granulocytes

PETER ELSBACH, SUSAN BECKERDITE, PENELOPE PETTIS, AND RICHARD FRANSON

Department of Medicine, New York University School of Medicine, New York, New York 10016

Received for publication 18 December 1973

Escherichia coli incubated in balanced salt solution with glucose as a carbon source but no nitrogen source exhibit a marked step-up of macromolecular synthesis when various non-bactericidal tissue extracts, or fractions thereof, are added. When disrupted granulocytes that cause rapid loss of viability are added, a step-up is also observed; i.e., incorporation of labeled precursors into ribonucleic acid is stimulated more than 15-fold, and incorporation into protein and deoxyribonucleic acid about twofold. This stimulation of macromolecular synthesis is still evident 30 min after more than 95% of the *E. coli* have lost their ability to multiply. Stimulation by disrupted granulocytes of [^{14}C]leucine incorporation into *E. coli* protein occurs over a wide range of leucine concentrations but is usually eliminated by adding a Casamino Acids mixture or another more complete medium. The substance(s) in tissue homogenates that trigger step-up is heat stable and dialyzable. Thus, *E. coli* exposed to the bactericidal and digestive components of disrupted granulocytes and no longer capable of division maintain their ability to regulate macromolecular synthesis in response to changes in nutritional conditions for at least 1 h.

Recently we have shown that macromolecular synthesis by *Escherichia coli* continues for at least 1 h after these microorganisms have been ingested and killed in vitro by polymorphonuclear leukocytes. In contrast, under similar conditions of rapid killing, other microorganisms such as *Micrococcus lysodeikticus* and *Bacillus megaterium* almost immediately lose their ability to carry out integrated biochemical activity (3). We attributed the difference in the fates of the biochemical apparatus of these microorganisms to differences in the extent of structural disorganization during phagocytosis; i.e., the envelope of *E. coli* remains grossly intact and thereby protects the metabolic machinery. This contention is supported by the finding that degradation of bacterial macromolecular constituents is only partial (1-3, 6) and by unpublished observations indicating that killing of *E. coli* by granulocytes does not result in release of soluble beta-galactosidase.

It has been well established that the nutritional conditions that determine the rate of growth strongly influence the rate of bacterial macromolecular synthesis (5). Thus, upon transfer of a bacterial population from a deficient medium to an enriched one, a "step-up" and, upon transfer from an enriched medium to a nutritionally poor medium, a "step-down" in macromolecular synthesis ensue. Similarly, in more specific terms, adding of an appropriate inducer results in the drastically increased synthesis of a particular enzyme. These shifts in rate of macromolecular synthesis are exceedingly rapid and reflect an extraordinarily effective metabolic adjustment to environmental changes.

Our recent demonstration of persistence of inducibility of beta-galactosidase during phagocytosis (3) already provides evidence that at least some control mechanisms remain operative in *E. coli* being killed by granulocytes. We extended these observations and now show that *E. coli* during rapid killing by disrupted granulocytes not only continue macromolecular synthesis after most organisms can no longer divide, but also remain capable of a general step-up in macromolecular synthesis in response to nutrients present in the disrupted granulocytes.

MATERIALS AND METHODS

The procedures have recently been published (3) and will only be described in outline.

Granulocytes. Granulocytes were obtained from sterile peritoneal exudates produced in rabbits. Ho-

mogenates were prepared from concentrated suspensions of granulocytes in distilled water by using a Potter-type homogenizer with a motor-driven Teflon pestle. The effectiveness of the disruptive procedure was monitored by phase-contrast microscopy. Fractions of the disrupted granulocytes were prepared by centrifugation as indicated in the text.

Growing and radioisotopic labeling of bacteria. *E. coli* W was grown, and where indicated labeled with [1-^{14}C]leucine during growth, in minimal medium buffered with triethanolamine (TEA growth medium) at pH 7.9 as previously reported (7).

Incubation procedure. *E. coli* and disrupted granulocytes (or fractions thereof) were incubated in a ratio of 20:1, unless indicated otherwise. Typical incubation mixtures contained 5×10^8 *E. coli* and 2.5×10^7 disrupted granulocytes in a total volume of 0.5 ml of sterile Hanks solution, buffered at pH 7.4 with tris(hydroxymethyl)aminomethane-maleate in a final concentration of 0.04 M. Although disrupted granulocytes are essentially incapable of incorporating labeled precursors into cellular protein or other macromolecules, cycloheximide in a concentration of 0.5 mM was added to all incubation mixtures to exclude participation of the mammalian cellular biochemical apparatus in any protein synthesis.

Synthesis of bacterial protein, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) was determined by following, respectively, the appearance of [^{14}C]leucine (International Chemical and Nuclear Corp.), diluted to a specific activity of 5.8 mCi/mmol, of [2-^{14}C]thymidine (Amersham-Searle Co.), diluted to a specific activity of 20 mCi/mmol (1), and of [2-^{14}C]uracil (New England Nuclear Corp.), diluted to a specific activity of 2.2 mCi/mmol, into acid-precipitable material. These precipitates were prepared by transferring 0.1-ml samples into test tubes that contained 2 ml of ice-cold 10% trichloroacetic acid. Filtering, washing, and counting of the radioactivity of the acid precipitates were carried out as recently described (3).

Viability of bacterial populations was examined by using standard culture procedures.

RESULTS

It has been observed by Hirsch that *E. coli* are as readily killed in vitro by disrupted granulocytes as by intact polymorphonuclear leukocytes (4). Similarly, in these experiments after 15 min of incubation of 5×10^8 *E. coli* with 2.5×10^7 disrupted granulocytes, 10% or less of the *E. coli* population could still divide, and after 30 min fewer than 5% were capable of multiplication. Nevertheless, leucine incorporation into *E. coli* protein continues, as does the synthesis of beta-galactosidase upon addition of inducer (3). After preincubation of *E. coli* and broken leukocytes for 0, 15, or 30 min (so that different degrees of destruction had taken place before addition of labeled leucine), incorporation of radioactivity into bacterial protein did not fall below control levels until after 30 min of preincubation (Fig. 1). In fact, it appears that labeling of *E. coli* protein was stimulated after 0 and 15 min of preincubation, even though degradation of previously labeled protein was increased during killing by the disrupted granulocytes (Table 1). Stimulation of leucine incorporation by bactericidal concentrations of disrupted granulocytes (in the presence of cycloheximide) did indeed occur. This stimulation depended upon the ratio of granulocytes and bacteria (Fig. 2 and 3). Leucine incorporation by 5×10^8 *E. coli* was enhanced at granulocyte concentrations ranging from 5×10^6 to 2.5×10^7 per 0.5 ml of incubation mixture (Hanks medium) (Fig. 2). Higher concentrations of granu-

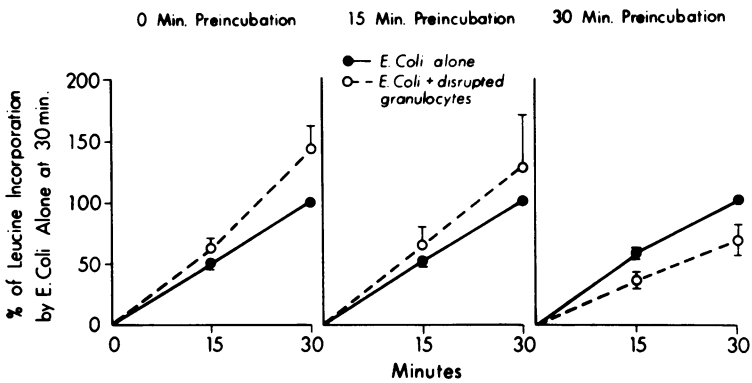

FIG. 1. *Effect of preincubation with disrupted granulocytes on [^{14}C]leucine incorporation by E. coli. Incubations were carried out in Hanks solution as described in Materials and Methods. Results are expressed as percentage of leucine incorporation by E. coli alone at 30 min and presented as mean ± standard error of the mean (dashes). Determination of colony-forming units after 30 min of incubation with granulocyte homogenates showed less than 5% surviving organisms. Preincubation for 15 and 30 min (total incubation, 45 and 60 min) caused little additional killing.*

TABLE 1. *Net loss of acid-precipitable radioactivity from [1-^{14}C]leucine-, [2-^{14}C]uracil-, [2-^{14}C]thymidine-labeled E. coli constituents during killing of E. coli by disrupted granulocytes*[a]

Incubation time (min)	Label					
	Leucine		Uracil		Thymidine	
	E. coli alone	*E. coli* plus granulocytes	*E. coli* alone	*E. coli* plus granulocytes	*E. coli* alone	*E. coli* plus granulocytes
0	100	100	100	100	100	100
15	99.8 ± 1.1 (5)	77.4 ± 8.4 (3)		88.6 ± 10.5 (3)		86.4 ± 5.1 (4)
30	96.2 ± 2.2 (8)	82.5 ± 6.6 (5)		85.2 ± 10.3 (3)		83.0 ± 4.4 (4)
60	94.5 ± 1.7 (10)	80.4 ± 5.1 (5)	88.4 ± 6.4 (6)	79.9 ± 8.5 (3)	86.3 ± 5.8 (3)	80.1 ± 2.7 (4)
120			92.0 ± 9.8 (3)	72.5 ± 8.1 (3)	80.6 ± 3.7 (3)	77.1 ± 3.6 (4)
300					79.3 ± 4.3 (3)	73.7 ± 1.3 (3)

[a] Labeling of *E. coli* during growth and determination of acid-precipitable radioactivity were carried out as recently described (3). All samples contained 5 × 10^8 *E. coli* and 2.5 × 10^7 disrupted granulocytes in 0.5 ml of incubation mixture. Loss of acid-precipitable radioactivity (percent of zero-time values) is presented as mean ± standard error of the mean of the indicated number of observations (in parentheses).

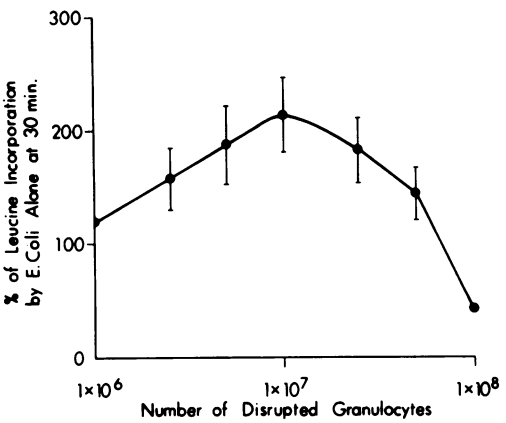

FIG. 2. *Effect of number of disrupted granulocytes on stimulation of [^{14}C]leucine incorporation by E. coli. Results are expressed as in Fig. 1. The number of surviving organisms, on the average, was 50% with 10^6 disrupted granulocytes, 4% with 4 × 10^6, 16% with 1.5 × 10^7, 3% with 3 × 10^7, and less than 1% with 6 × 10^7 granulocytes after incubation for 30 min.*

locytes may depress labeling of *E. coli* protein. The effect of increasing numbers of bacteria on stimulation of *E. coli* protein synthesis by a fixed number of disrupted granulocytes is depicted in Fig. 3. Stimulation was as pronounced at 30 min as at 60 min over a range of *E. coli* concentrations from 5 × 10^7 to 1 × 10^9, but was no longer evident at 5 × 10^9 *E. coli* per 2.5 × 10^7 granulocytes.

The stimulatory effect of disrupted granulocytes on leucine incorporation by *E. coli* is seen over a 20-fold range of leucine concentrations. At 1.3 mM, leucine incorporation approached a plateau both in the absence and in the presence of disrupted granulocytes (Fig. 4).

Figure 5 shows the effect of bactericidal concentrations of disrupted granulocytes on incorporation of [^{14}C]thymidine and [^{14}C]uracil into nucleic acids of *E. coli* in Hanks medium. Stimulation of thymidine incorporation was of approximately the same magnitude (about two-fold at 30 min) as shown above for leucine incorporation; uracil incorporation, on the other hand, was increased many fold at 30 min.

It should be noted that increased incorporation of labeled thymidine and uracil occurs in the face of a moderately enhanced loss of radioactivity from previously labeled *E. coli* DNA and RNA in the presence of disrupted

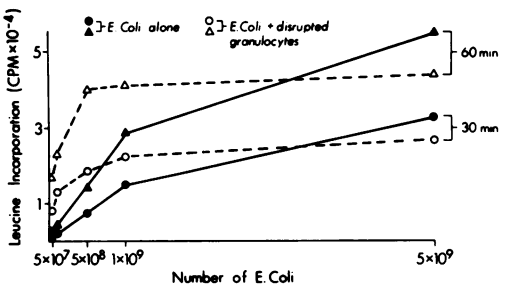

FIG. 3. *Effect of number of E. coli on stimulation by disrupted granulocytes of [^{14}C]leucine incorporation by E. coli. Leucine incorporation into E. coli protein is shown as counts per minute in acid-precipitable material (3).*

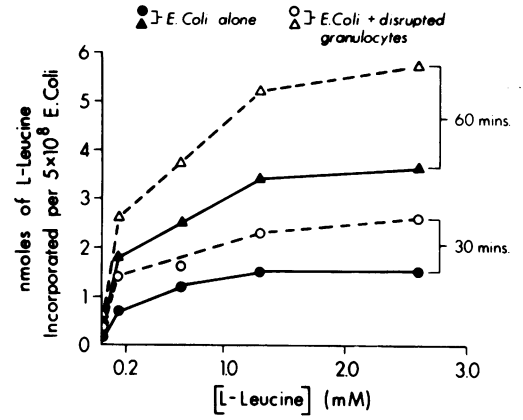

FIG. 4. *Effect of leucine concentration on stimulation by disrupted granulocytes of leucine incorporation by E. coli. Leucine incorporation is expressed as nanomoles incorporated per 5 × 10⁸ E. coli.*

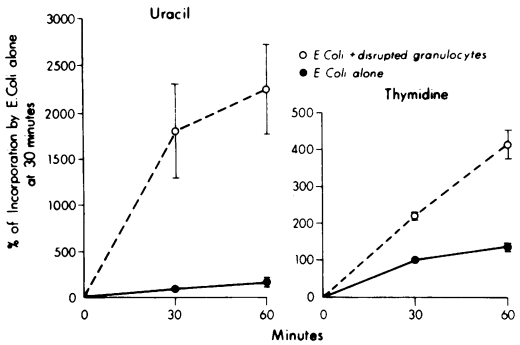

FIG. 5. *Effect of disrupted granulocytes on incorporation of [¹⁴C]thymidine and [¹⁴C]uracil by E. coli. The experiments were carried out as described in Materials and Methods and in a recent publication (3). Results are expressed as in Fig. 1.*

granulocytes (Table 1). The much greater stimulatory effect on uracil incorporation than on leucine or thymidine incorporation closely resembles the step-up phenomenon described by Neidhardt and Magasanik (5). The following observations support this contention. (i) In a supplemented medium, uracil incorporation was no longer increased in the presence of granulocytes. In fact, in many experiments a reduction was observed in incorporation of uracil or leucine of approximately 30% (Table 2). (ii) Other tissue homogenates (brain, kidney, liver) that are not bactericidal, and supernatant fractions of granulocyte homogenates obtained by centrifugation for 1 h at 100,000 × *g* that do not have bactericidal activity, also stimulated in Hanks solution but not in the enriched medium. Thus, the stimulatory effects on macromolecular synthesis by *E. coli* did not depend on some damage exerted by the granulocyte, such as a change in permeability of the *E. coli* envelope, associated with killing. On the other hand, until high cell concentrations were reached the damage that had to accompany killing did not alter the stimulatory effect of bactericidal concentration of disrupted granulocytes when compared with a non-bactericidal supernatant fraction (Fig. 6).

We have not yet identified the substance(s) that causes the stimulation by tissue homogenates. The effect was seen after boiling and even after autoclaving the tissues or fractions prepared from them. This finding and the presence of the activity in all tissues examined so far suggested a nonspecific component(s) probably of small molecular size. This is indeed the case, as indicated by the observation that equilibrium dialysis for 2 h of an autoclaved supernatant fraction of a granulocyte homogenate (obtained by centrifugation at 20,000 × *g* for 20 min) resulted in almost complete equilibration of the stimulatory agent(s) (Table 3). Exhaustive dialysis resulted in loss of activity.

Among a number of substances added to

TABLE 2. *Effect of an autoclaved supernatant fraction of disrupted granulocytes on [2-¹⁴C]uracil incorporation by E. coli in a nutrient-poor or a supplemented medium*[a]

Medium	Acid-precipitable counts/min	%
Hanks solution[b]	2,060	100
Hanks solution plus granulocyte fraction (1.25 × 10⁷)	7,480	363
Hanks solution plus granulocyte fraction (2.5 × 10⁷)	14,060	683
Hanks solution plus TEA growth medium[c]	31,290	1,520
Hanks solution plus TEA growth medium plus granulocyte fraction (1.25 × 10⁷)	18,730	910
Hanks solution plus TEA growth medium plus granulocyte fraction (2.5 × 10⁷)	21,210	2,030

[a] Incubation mixtures of 0.25 ml contained 2.5 × 10⁸ *E. coli* and 1.25 × 10⁷ or 2.5 × 10⁷ cell equivalents of a heat-treated (120 C at 2.7 kg/cm²) 20,000 × g (20 min) supernatant fraction of disrupted granulocytes. Mixtures were preincubated for 30 min before adding 10⁵ disintegrations/min [2-¹⁴C]uracil (50 μg). Incubation was carried out for 30 min, and the reaction was stopped by adding 2 ml of ice-cold 10% trichloroacetic acid.

[b] Final concentration of glucose (4 mM), Ca^{2+} (0.8 mM), Mg^{2+} (0.8 mM).

[c] Final concentration of $(NH_4)_2SO_4$ (80 mg per 100 ml), lactate (200 mg per 100 ml), Casamino Acids (80 mg per 100 ml).

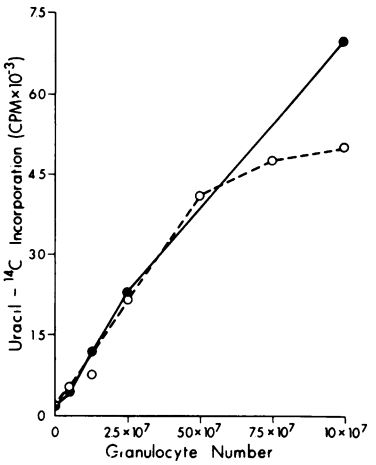

FIG. 6. *Comparison of stimulation of [^{14}C]uracil incorporation into E. coli RNA by disrupted granulocytes (bactericidal) and by a granulocyte supernatant fraction (non-bactericidal). The supernatant fraction was prepared by centrifugation of disrupted granulocytes at 20,000 × g for 20 min. This fraction was exposed to 120 C and a pressure of 2.7 kg/cm² for 15 min. Disrupted granulocytes, O; supernatant fraction, ●. For number of surviving E. coli after 30 min of incubation with increasing concentration of disrupted granulocytes, see legend to Fig. 2.*

TABLE 3. *Dialyzability of the stimulatory activity in an autoclaved supernatant fraction of disrupted granulocytes on uracil incorporation by E. coli*

E. coli treatment	Acid precipitable counts/min after incubation for:	
	30 min	60 min
Alone in Hanks solution	225	665
Plus granulocyte fraction (0.05 ml)	5,470	6,865
Plus granulocyte fraction (0.1 ml)	8,685	9,025
After dialysis for 2 h[a]		
Plus 0.1 ml of contents of chamber A	5,850	6,630
Plus 0.1 ml of contents of chamber B	5,040	5,255

[a] Dialysis was carried out in a Plexiglas cell (Arthur H. Thomas Co., Philadelphia, Pa.) consisting of two 1-ml compartments separated by a single layer of dialysis tubing (average pore diameter, 4.8 nm). At time zero, compartment A received 1 ml of autoclaved supernatant fraction and compartment B received 1 ml of tris(hydroxymethyl)aminomethane-maleate buffer (pH 7.4). After shaking for 2 h at 37 C, 0.1-ml samples were taken for assay of stimulatory effect on [^{14}C]uracil incorporation into *E. coli* RNA as described in the legend of Fig. 5. Identical results were obtained in the presence of 100 mM KCl.

Hanks medium, including Mg^{2+} (4 and 20 mM), Ca^{2+} (4 and 20 mM), lactate (0.2 g per 100 ml), NH_4SO_4 (0.1 g per 100 ml) and Casamino Acids (0.2 g per 100 ml), only the amino acids markedly stimulated uracil incorporation by *E. coli*. In numerous experiments, the stimulatory effect of disrupted granulocytes and of non-bactericidal fractions thereof was no longer seen in the presence of added amino acids. However, on occasion we have observed an additive stimulatory effect of Casamino Acids or complete TEA growth medium plus an autoclaved supernatant fraction of a granulocyte homogenate (tested in amounts representing 2.5×10^7 and 5×10^7 granulocyte equivalents). Stimulation may be caused, therefore, by dialyzable tissue components other than or in addition to amino acids or small peptides.

DISCUSSION

The transfer of a population of bacteria from a nutrient-deficient to an enriched medium is very rapidly followed by a step-up of macromolecular synthesis (5), presumably reflecting preparation for growth under more favorable conditions.

That this step-up phenomenon was also seen in these experiments when the incubation medium (Hanks balanced salt solution plus glucose) was supplemented with tissue homogenate is not surprising. It is unexpected, however, that fractions of granulocytes that are rapidly bactericidal permit the *E. coli* to make as immediate and extensive a metabolic adjustment as observed with fractions devoid of bactericidal activity. (We have also observed stimulation of leucine incorporation in deficient medium by other *E. coli* strains and other gram-negative microorganisms exposed to bactericidal concentrations of granulocyte homogenates.)

We have recently reported that during phagocytosis of *E. coli* by intact granulocytes, macromolecular synthesis, including synthesis of beta-galactosidase upon addition of inducer, continues for at least 1 h after more than 95% of the *E. coli* can no longer multiply (3). We have provided evidence suggesting that this preservation of functional activity reflects the inability of the granulocyte to rapidly and extensively degrade the ingested *E. coli* envelope. Thus, during the initial hour the metabolic machinery of *E. coli* remains protected and, to a considerable extent, intact. The ability of *E. coli*, killed by disrupted granulocytes, to express previously repressed genetic loci (as evidenced by induction of beta-galactosidase) and to step up macromolecular synthesis, apparently because of

addition of simple nutrients, provides further evidence that granulocytes can cause loss of ability to multiply without gross structural and biochemical impairment.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AM 05472 from the National Institute of Arthritis, Metabolism and Digestive Diseases.

P. E. is a career scientist of the Health Research Council of the City of New York (contract I-379). R. F. is a recipient of Public Health Service postdoctoral fellowship I-F-02 AM 54970-1 from the National Institute of Arthritis, Metabolism and Digestive Diseases.

LITERATURE CITED

1. Cohn, Z. A. 1963. The fate of bacteria within phagocytic cells. I. The degradation of isotopically labeled bacteria by polymorphonuclear leukocytes and macrophages. J. Exp. Med. **117**:27-42.
2. Elsbach, P., J. Goldman, and P. Patriarca. 1972. Phospholipid metabolism by phagocytic cells. VI. Observations on the fate of phospholipids of granulocytes and ingested *Escherichia coli* during phagocytosis. Biochim. Biophys. Acta **280**:33-44.
3. Elsbach, P., P. Pettis, S. Beckerdite, and R. C. Franson. 1973. Effects of phagocytosis by rabbit granulocytes on macromolecular synthesis and degradation in different species of bacteria. J. Bacteriol. **115**:490-497.
4. Hirsch, J. G. 1956. Phagocytin: a bactericidal substance from polymorphonuclear leukocytes. J. Exp. Med. **103**:589-611.
5. Neidhardt, F. C., and B. Magasanik. 1960. Studies on the role of riboncucleic acid in the growth of bacteria. Biochim. Biophys. Acta **42**:99-116.
6. Patriarca, P., S. Beckerdite, P. Pettis, and P. Elsbach. 1972. Phospholipid metabolism by phagocytic cells. VII. The degradation and utilization of phospholipids of various microbial species by rabbit granulocytes. Biochim. Biophys. Acta **280**:45-56.
7. Wurster, N., P. Elsbach, J. Rand, and E. J. Simon. 1971. Effects of levorphanol on phospholipid metabolism and composition in *Escherichia coli*. Biochim. Biophys. Acta **248**:282-292.