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THE USE OF A PHOSPHOLIPASE A-LESS *ESCHERICHIA COLI* MUTANT TO ESTABLISH THE ACTION OF GRANULOCYTE PHOSPHOLIPASE A ON BACTERIAL PHOSPHOLIPIDS DURING KILLING BY A HIGHLY PURIFIED GRANULOCYTE FRACTION

J. WEISS and P. ELSBACH *

Department of Medicine, New York University School of Medicine, New York N.Y. 10016 (U.S.A.)

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Summary

Phospholipase A_2 present in a highly purified, potently bactericidal, fraction from rabbit granulocytes produces net bacterial phospholipid degradation during killing of a phospholipase A-less strain of *Escherichia coli*. In the wild-type parent strain phospholipid breakdown is caused not only by the action of phospholipase A_2 but also by phospholipase A_1, indicating activation of the most prominent phospholipase of *E. coli*. This activation occurs as soon as the bacteria are exposed to the granulocyte fraction. Phospholipid breakdown by both phospholipases A is dose dependent but reaches a plateau after 30—60 min and at higher concentrations of the fraction.

Phospholipid degradation is accompanied in both strains by an increase in permeability to actinomycin D that is also dose dependent. Even though net hydrolysis of phospholipids is greater in the parent strain than in the mutant, the increase in permeability is the same in the two strains.

The addition of 0.04 M Mg^{2+}, after the effects on phospholipids and permeability have become manifest, initiates in both strains the restoration of insensitivity to actinomycin D, the net resynthesis of phospholipids, and the disappearance of monoacylphosphatides and the partial disappearance of free fatty acids that had accumulated. Loss of ability to multiply is not reversed by Mg^{2+} in either strain. Less than 5 μg of granulocyte fraction causes loss of viability of from 90 to 99% of 1×10^8 microorganisms of both strains. However, at lower concentrations the parent strain is considerably more sensitive to the bactericidal effect of the granulocyte fraction than the mutant strain.

* To whom correspondence should be addressed.

Introduction

Rapid killing of *E. coli* W by highly purified fractions from rabbit polymorphonuclear leukocytes is accompanied by almost immediate alterations of the bacterial envelope, namely a discrete increase in permeability and degradation of phospholipids [1,2]. These purified fractions contain phospholipase A_2 activity. However, since *E. coli* also possess a phospholipid-degradative apparatus [3—5], that can become activated under conditions adverse to the microorganism [6—8] the participation of the granulocyte phospholipase in the attack on the membrane phospholipids was uncertain.

In order to determine whether the phospholipid degradation that accompanies killing by the purified granulocyte graction is wholly or in part caused by the phospholipase A_2 activity of the granulocyte fraction, or attributable to activation of endogenous phospholipases, we compared the effects of the granulocyte fraction on an *E. coli* mutant devoid of demonstrable phospholipase activity with those on a parent (K_{12}) *E. coli* strain endowed with a phospholipid-degradative apparatus typical for *E. coli* [3,4]. We find that killing of both strains by the granulocyte fraction is accompanied by phospholipid hydrolysis and increased permeability. Whereas in the mutant phospholipid hydrolysis can be ascribed almost exclusively to phospholipase A_2 action, hydrolysis of phospholipids in the parent strain is also the consequence of phospholipase A_1 activity. These results indicate that the granulocyte phospholipase A_2 does attack the membrane phospholipids of intact *E. coli* and that activation of endogenous phospholipase A_1 of the bacteria can further contribute to hydrolysis.

Materials and Methods

Preparation of granulocytes. Polymorphonuclear leukocytes were obtained from overnight, sterile peritoneal exudates produced in rabbits by injection of glycogen in physiological saline as described previously [9], except that no heparin was added to the collection flask. More than 95% of the cells were granulocytes as judged by differential cell count. The cells were sedimented by centrifugation at $50 \times g$ for 10 min and resuspended in the desired medium.

Preparation of purified fractions. Purification of sulfuric acid extracts of polymorphonuclear leukocytes by carboxymethyl-Sephadex chromatography was carried out as recently described in detail [1]. The extent of purification achieved has ranged in different preparations from 200- to 400-fold with a yield from 40 to 80%, using biological activity in whole homogenate as reference [1]. These preparations from hereon will be referred to as granulocyte fraction.

Bacteria. E. coli strains S_{15} and S_{17} (F^{-1} thi^1 leu^1 thr^1 lac^1 mel^1) were kindly donated by Prof. S. Nojima, Department of Chemistry, National Institute of Health, Tokyo, Japan. The absence of both detergent-resistant and detergent-sensitive phospholipase A activities in S_{17} [10,4] was confirmed by heat (60°C) treatment and spheroplast formation, procedures which activate the bacterial phospholipases [11]. Both strains were grown in minimal medium buffered with triethanolamine at pH 7.75—7.9 [7]. The bacteria used were obtained from overnight cultures that were transferred to fresh medium and subcultured

for approximately 3.5 h at 37°C. At this time the bacteria were sedimented by centrifugation at 10 000 × *g* for 10 min and resuspended in sterile isotonic saline in the desired concentration.

Assay for permeability changes. The effect of the purified granulocyte fraction on the permeability of the envelope of *E. coli* was examined by determining the susceptibility of *E. coli* to actinomycin D, an agent that normally does not cross *E. coli's* permeability barrier [12]. An effect on the microbial barrier was measured by determining the effect of the granulocyte fraction on [^{14}C]-leucine incorporation into bacterial protein in the presence and absence of actinomycin D as described previously [13]. A typical incubation mixture contained 10^8 *E. coli* (S_{15} or S_{17}) in a total volume of 0.25 ml of sterile physiological saline that also contained 10 μmol of Tris/maleate buffer at pH 7.5, 25 μl of Hank's solution (Hank's balanced salt solution (without phenol red) Microbiological Associates, Inc., Bethesda, Md.), 250 μg of casamino acid (Difco Laboratories, Detroit, Mich.) and the granulocyte fraction in the concentration indicated. L-[1-^{14}C]leucine (0.063 μCi, 0.13 mM) (ICN Corp., Chemical and Radioisotopes Div., Irvine, Calif.) was added and the samples were incubated at 37°C for 30 min in the presence or absence of 12.5 μg actinomycin D. The reactions were stopped by the addition of 3.0 ml of ice-cold 10% trichloracetic acid and the mixtures were filtered and counted as recently described [13].

Labeling of E. coli phospholipids. Bacterial phospholipids were labeled during growth in subculture. Aliquots of an overnight culture of either strain grown in triethanolamine medium as described above were diluted 1 : 10 in fresh medium. After incubation for 2 h at 37°C, aliquots of the subculture were transferred to flasks containing 0.2 μCi/ml of [1-^{14}C]palmitic acid or [1-^{14}C]oleic acid (specific activity 59.9 Ci/mol; Amersham Searle Corp., Arlington Heights, Ill.) complexed with 0.02% bovine serum albumin (fatty acid poor, Fraction V, Pentex, Miles Research Products, Elkhart, Ind.). After incubation for 30 min at 37°C, the bacteria were sedimented by centrifugation at 10 000 × *g* for 10 min, resuspended in fresh triethanolamine medium, and reincubated for 30 min to permit the remaining unincorporated labeled precursor to be incorporated. The labeled bacteria were washed with 2% albumin and resuspended in saline.

The position of the incorporated labeled fatty acids was determined as previously described [14]. Lipids, extracted from the two strains, labeled during growth, were hydrolyzed by treatment with boiled *Vipera russelli* venom phospholipase A_2. Of the subsequently isolated radioactive products of hydrolysis, more than 90% were monoacylphosphatides in the case of palmitic acid-labeled *E. coli* and more than 95% were free fatty acids in the case of oleic acid-labeled bacteria.

Lipid extraction and fractionation. Lipids were extracted according to the procedure of Bligh and Dyer [15]. The removed aqueous methanolic upper phase was washed once with 0.5 vol. of $CHCl_3$ to optimize recovery of monoacylphosphatides. The combined $CHCl_3$ extracts were dried under a nitrogen stream, redissolved in 0.1 ml $CHCl_3/CH_3OH$ (2 : 1) and transferred to commercial silica gel F 254 plates (Brinkman Instruments, Westbury, N.Y.). Monoacylphosphatides, diacylphosphatides and fatty acids were separated in a solvent system consisting of chloroform/methanol/distilled water/glacial acetic acid

(65 : 25 : 4 : 1, v/v). Lipid species were identified by comparison of R_F with that of authentic standards after visualization following exposure of the plates to iodine vapors. Liquid scintillation counting of thin-layer fractions scraped off the plates into counting vials was carried out as described previously [16].

Viable counts. At the indicated times, 10 μl samples were taken from the incubation suspension, serially diluted in sterile isotonic saline and plated on nutrient agar. After incubation overnight at 37°C the number of colony forming units on the plates was determined.

Results

Effect of granulocyte fraction on viability of S_{15} and S_{17}. Fig. 1 shows that greater than 90% killing of 10^8 organisms occurs during incubation of either strain with less than 5 μg of granulocyte fraction. However, the parent strain is more sensitive to this bactericidal effect. Thus, 1—2 μg of the granulocyte fraction has little or no effect on the viability of the mutant strain (S_{17}) but causes a reduction in colony formation by the parent strain (S_{15}) of at least one log.

Degradation of E. coli phospholipids during incubation with granulocyte fraction. To determine phospholipid degradation the bacterial phospholipids were radioactively labeled during growth either with [1-^{14}C]palmitic acid or with [1-^{14}C]oleic acid. In both strains more than 90% of [^{14}C]palmitic acid radioactivity is incorporated into the 1-position of the major *E. coli* phospholipids, phosphatidylethanolamine and phosphatidylglycerol; of [^{14}C]oleic acid more than 90% appears in the 2-position [2,5]. Hence analysis of the labeled products of hydrolysis (free fatty acids and monoacyl phosphatides) should reveal whether degradation is mainly attributable to phospholipase A_1 or to phospholipase A_2 activity. This prediction only holds of course if deacylation of the formed monoacylphosphatides by lysophospholipase activity does not occur to any great extent. In the present and previous studies of phospholipid

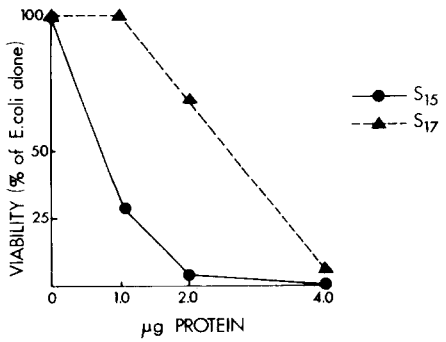

Fig. 1. Effect of increasing concentrations of granulocyte fraction on viability of *E. coli* (S_{15} or S_{17}). 1×10^8 E. coli (S_{15} or S_{17}) were incubated at 37°C with increasing concentrations of granulocyte fraction in the standard incubation mixtures (10 μmol of Tris/maleate buffer at pH 7.5; 25 μl of Hanks' solution; 250 μg of casamino acids, in a total volume of 0.25 ml of sterile physiological saline). After incubation for 30 min, an aliquot was taken and colony-forming units were determined as described in Materials and Methods. The solid line represents incubation of S_{15}, the broken line, of S_{17}. The results shown represent the mean of four closely similar experiments.

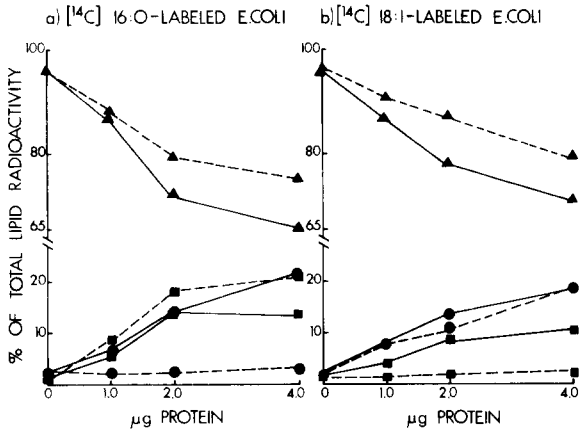

Fig. 2. Effect of increasing concentrations of granulocyte fraction on degradation of *E. coli* (S_{15} or S_{17}) phospholipids. Of *E. coli* populations (S_{15}, solid line; S_{17}, broken line) labeled during growth with (a) [1-^{14}C]palmitic acid or (b) [1-^{14}C]oleic acid as described in Materials and Methods, 1×10^8 microorganisms were incubated at 37°C for 30 min with increasing concentrations of granulocyte fraction in the standard incubation mixture. Amounts of diacylphosphatides ▲; monoacylphosphatides ■; and free fatty acids ●, are shown as percent of total lipid radioactivity. Results shown represent the mean of three experiments. Total lipid radioactivity per sample ranged from 10 000—25 000 cpm in different experiments. In a given experiment recovery of radioactivity was approximately the same for each sample. Individual samples contained approximately 2 nmol of phospholipid.

breakdown during killing of *E. coli* by granulocytes or granulocyte fractions, formation of labeled diglyceride has never been detected, indicating that phospholipase C activity does not contribute to hydrolysis.

No detectable net phospholipid hydrolysis occurs during incubation of the two prelabeled strains in the absence of granulocyte fraction (not shown). In the presence of granulocyte fraction, however, breakdown of the phospholipids of both strains is readily apparent (Fig. 2). The labeled products formed during hydrolysis of [^{14}C]palmitate and [^{14}C]oleate prelabeled S_{15} and S_{17} exposed to granulocyte fraction are different. Radioactive free fatty acids as well as monoacylphosphatides accumulate during incubation of [^{14}C]palmitate labeled S_{15}, but the radioactive products formed during incubation of [^{14}C]palmitate labeled S_{17} are almost exclusively monoacylphosphatides (Fig. 2a). The degradation of [^{14}C]oleate prelabeled S_{15} again leads to accumulation of both radioactive free fatty acids and monoacylphosphatides, but in the case of [^{14}C]oleate labeled S_{17} almost solely to the appearance of labeled free fatty acids (Fig. 2b). Thus, whereas hydrolysis of the phospholipid in S_{17} is almost totally restricted to the 2-acyl position, the phospholipids in S_{15} are degraded at both acyl locations. These results are consistent with the absence of endogenous phospholipase A_1 activity in the mutant strain [4,10] and suggest action of the exogenous granulocyte phospholipase A_2 on the *E. coli* phospholipids. The greater net hydrolysis in the parent strain is largely attributable to the additional breakdown at the 1-acyl position. In both organisms, exposure to higher concentrations of granulocyte fraction causes more extensive phospholipid degradation. In S_{15} hydrolysis at both fatty acid positions is increased. However, at higher concentrations of granulocyte fraction, accumulation of labeled monoacylphos-

phatides is less pronounced than of free fatty acids, indicating further deacylation.

The time course of net degradation of the envelope phospholipids of S_{15} and S_{17} during exposure to 3.5 μg of granulocyte fraction is shown in Fig. 3. Degradation reaches a plateau in 30 min. In S_{15} (top portion of Fig. 3), labeled either with [^{14}C]palmitic acid or with [^{14}C]oleic acid, radioactive monoacylphosphatides accumulate during the first 15 min and decrease thereafter. Radioactive fatty acids continue to accumulate for an additional 15 min. A decrease in monoacylphosphatide radioactivity with a corresponding increase in radioactive free fatty acids is also apparent in [^{14}C]palmitic acid labeled S_{17} (bottom of Fig. 3) after 30 min, when net hydrolysis has stopped. Because the highly purified granulocyte fraction is devoid of detectable lysophospholipase activity (unpublished observations), it follows that bacterial lysophospholipase activity participates in degradation of envelope phospholipids of the parent strain as well as the phospholipase A-less mutant. These findings are consistent with the recent report of Doi and Nojima [17], showing that extracts of three mutants

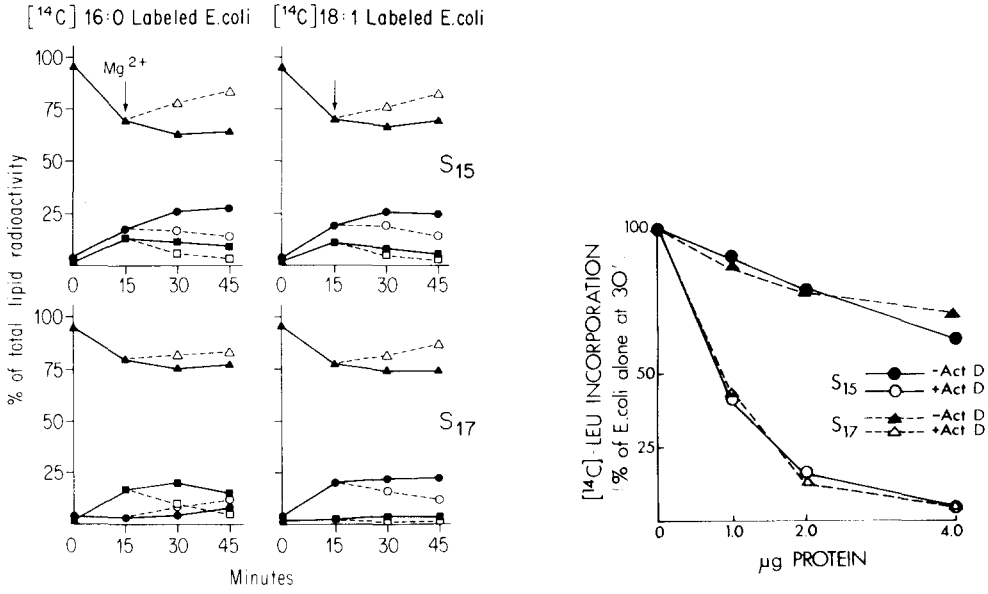

Fig. 3. Time course of degradation of *E. coli* (S_{15} or S_{17}) phospholipids during incubation with granulocyte fraction; effect of Mg^{2+}. Of labeled *E. coli* (S_{15} or S_{17}) (see Materials and Methods and legend to Fig. 2) 1×10^8 microorganisms were incubated at 37°C with 3.5 μg of granulocyte fraction in the standard incubation mixture. $MgCl_2$ (40 mM final concentration) was added where indicated. The solid line represents incubation before Mg^{2+} addition, the broken line incubation after Mg^{2+} addition. For other symbols and explanations see legend of Fig. 2. Results are expressed as percent of total lipid radioactivity and represent the mean of two experiments.

Fig. 4. Effect of increasing concentrations of granulocyte fraction on sensitivity of *E. coli* (S_{15} or S_{17}) to actinomycin D. Of strain S_{15} (solid line) or S_{17} (broken line) 1×10^8 organisms were incubated with increasing concentrations of granulocyte fraction in the presence (open symbols) and absence (closed symbols) of actinomycin D as described in Materials and Methods. Incorporation of [^{14}C]leucine into cold trichloroacetic acid-precipitable material is expressed as percent of incorporation by *E. coli* incubated alone for 30 min (4000 cpm; 4 nmol). Results shown represent the mean of four closely similar experiments.

of *E. coli* lacking phospholipase A contain lysophospholipase activity towards micellar substrates.

Effect of granulocyte fraction on permeability of the envelopes of S_{15} and S_{17}. Like other strains of *E. coli* [12,18] both S_{15} and S_{17} are normally impermeable to the antibiotic actinomycin D. Thus, during incubation with the bacteria, the drug cannot interact with the bacterial DNA and therefore does not inhibit macromolecular (including protein) biosynthesis. However, when either strain is exposed to the granulocyte fraction, incorporation of [^{14} C]leucine into cold trichloroacetic acid-precipitable material, only slightly reduced in the absence of actinomycin D, is markedly reduced in its presence (Fig. 4). This reflects the increased permeability of the microbial envelope to the antibiotic [18]. Incubation of either strain with higher concentrations of the granulocyte fraction increases the susceptibility of the bacteria to actinomycin D. Whereas the effect of the granulocyte fraction on net phospholipid hydrolysis is greater in the parent strain, its effect on the sensitivity of the two strains to actinomycin D is the same at all concentrations of granulocyte fraction tested.

Reversal of effects of granulocyte fraction on the envelopes of S_{15} and S_{17} during incubation with Mg^{2+}. We have shown that the effects of the granulocyte fraction on both the envelope phospholipids and permeability of *E. coli* W can be reversed during subsequent incubation of the treated bacteria with Mg^{2+}. Loss of ability to multiply is irreversible, however [2]. Restoration of the permeability barrier to actinomycin D appears to be linked to removal of the phos-

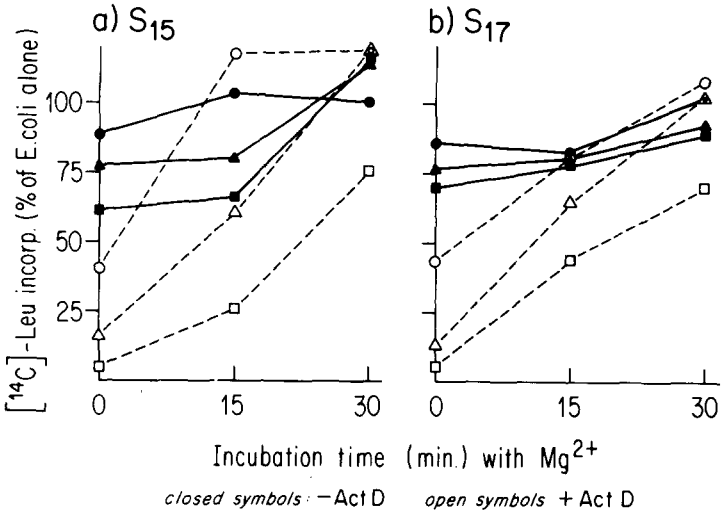

Fig. 5. Time course of restoration by *E. coli* S_{15} and S_{17} of permeability barrier to actinomycin D upon addition of Mg^{2+}. Of S_{15} (a) or S_{17} (b) 1×10^8 bacteria were preincubated for 15 min at 37°C with 1.0 μg (●, ○), 2.0 μg (▲, △) or 4.0 μg (■, □) of granulocyte fraction in the standard incubation mixture. Subsequently, $MgCl_2$ (40 mM final concentration) was added and incubation was continued for the indicated periods of time at 37°C before [^{14}C]leucine ± actinomycin D were added. Each smaple was then incubated at 37°C for 30 min and susceptibility to actinomycin D was measured in the usual way. Incorporation by *E. coli* incubated alone ranged from 3000—5000 cpm (3—5 nmol). Results shown represent the mean of at least three experiments.

pholipid breakdown products that accumulate during preincubation with the granulocyte fraction and the time required for recovery is increased in bacteria undergoing greater net phospholipid degradation before Mg^{2+} is added [2].

If indeed phospholipid metabolism and the breakdown and restoration of the permeability barrier to actinomycin D are linked in some way, the repair of the barrier upon addition of Mg^{2+} might be different in the parent and mutant strain, because the nature and extent of phospholipid degradation differ in the two strains (Figs. 2 and 3). We therefore compared the ability of S_{15} and S_{17}, after exposure to increasing concentrations of the granulocyte fraction, to restore their impermeability to actinomycin D during incubation with Mg^{2+}.

In Fig. 2 it was shown that net phospholipid breakdown increases in both strains as the amount of granulocyte fraction present during preincubation is increased. The inhibition of leucine incorporation at the end of preincubation (0 min in Fig. 5) is also dose dependent, as is the time required for recovery of insensitivity to actinomycin D after Mg^{2+} is added (Fig. 5). The time course of reversal by Mg^{2+} of the permeability effect is quite similar in the two strains at the three concentrations of granulocyte fraction tested, despite the greater net phospholipid degradation in the parent strain.

Fig. 3 shows that addition of Mg^{2+} after 15 min of exposure to granulocyte fraction also promptly results in reincorporation of the labeled products of hydrolysis into diacylphosphatides of both strains. This reincorporation is more marked in the parent strain (top portion of Fig. 3) than in the mutant strain (bottom portion), roughly matching the difference in degradation. In both strains labeled monoacylphosphatides disappear almost completely, possibly in part through continued deacylation. Radioactive free fatty acids are only partially reincorporated.

Discussion

These results demonstrate that the phospholipase A_2 present in a highly purified bactericidal fraction of rabbit granulocytes can hydrolyze the phospholipids of intact *E. coli*. The evidence on which this conclusion is based comes from the use of an *E. coli* mutant, isolated by Nojima et al. [3,4,10] which is devoid of demonstrable endogenous phospholipase A activity. This mutant does possess a lysophospholipase, but in highly purified form this enzyme does not hydrolyze diacylphosphatides [17]. Analysis of the labeled products of hydrolysis of the phospholipids of the mutant after exposure to granulocyte fraction indicates that hydrolysis is almost totally attributable to the action of a phospholipase A_2, consistent with the positional specificity of the granulocyte enzyme previously observed using other phospholipid substrates [19].

During the incubation of the parent strain, on the other hand, both 1-acyl and 2-acyl lysophosphatides accumulate, indicating that phospholipase A_2 as well as phospholipase A_1 contribute to hydrolysis. The parent strain is equipped with the full spectrum of phospholipid-splitting enzymes typical of wild-type *E. coli* [5]. However, the most prominent phospholipase activity of *E. coli* is a phospholipase A_1 [5,20]. It is activation of this enzyme that apparently is mainly responsible for the hydrolysis of *E. coli* phospholipids under adverse

conditions [8,11]. It seems probable therefore that phospholipid degradation in S_{15} is caused by the combined action of the exogenous granulocyte phospholipid A_2 and the bacterial enzyme which is activated upon exposure of the bacteria to the bactericidal granulocyte fraction.

It is of considerable interest that the granulocyte phospholipase A_2, under the conditions of these experiments, hydrolyzes the bacterial envelope phospholipids, because the phospholipids of *E. coli* strains with normal outer envelopes generally are not accessible to a broad range of pure phospholipases. Only after rather drastic alterations in the envelope, such as caused by defective outer membrane synthesis [21] or by EDTA treatment [22], does phospholipid degradation by exogenous phospholipases become manifest. Unpublished observations indicate that the phospholipase A_2 activity in the granulocyte fraction is a component of a cationic protein complex (approx. mol. wt. 50 000) that exhibits also bactericidal and envelope permeability increasing activities. Manipulations that cause dissociation of the complex into lower mol. wt. constituents cause loss of all biological activity towards intact *E. coli*. Thus, the phospholipase A_2 after dissociation of the complex, although readily demonstrable using other substrates, in low molecular weight form can apparently no longer reach the bacterial lipids. The permeability changes that accompany the phospholipid hydrolysis initiated by the granulocyte fraction may represent the membrane modification necessary to provide access to the membrane phospholipids. In any case, in order for these envelope effects to occur, the components of the purified fraction apparently cannot simply coexist as such, but must interact with the envelope in a prescribed association.

As soon as this interaction takes place with the envelopes of *E. coli* endowed with a complete phospholipid-splitting apparatus (*E. coli* S_{15} or W [2]), activation of the bacterial phospholipase A_1 becomes also manifest. This immediate and dose-dependent effect on an enzyme located in the outer membrane [5,23] may be related to the binding of active principle(s) of the granulocyte fraction to surface sites. Binding is required for expression of the biological effects of the fraction on *E. coli* [1]. The abrupt cessation, upon addition of Mg^{2+}, of both granulocyte and *E. coli* phospholipase A activity [2] further suggests a reversible physical-chemical perturbation by the granulocyte fraction of mainly the outer membrane. In support of this supposition Mg^{2+} also causes release of previously bound phospholipase A_2 activity [2], and morphological observations suggest that recognizable structural alterations do not extend beyond the outer membrane [2,24].

Net phospholipid degradation in *E. coli* killed by granulocytes or granulocyte fractions reaches a plateau after 30—60 min [2,25,26]. At concentrations of granulocyte fraction higher than shown in the present paper, net breakdown is not substantially increased. Among several possible explanations for the leveling off of envelope phospholipid breakdown we favor the following. The limited structural and functional disorganization that accompanies loss of viability has suggested to us that hydrolysis is confined to outer membrane phospholipids, presumably because the phospholipids of the cytoplasmic membrane are less accessible [27]. In addition, killing of *E. coli* by granulocyte preparations is accompanied by continued and, in fact, stimulated phospholipid synthesis [14]. Therefore, reincorporation of products of hydrolysis must also occur, reducing net degradation.

It remains to be established whether or not the close association, with respect to time, of an increase in envelope permeability and its return towards normal with added Mg^{2+} on the one hand, and respectively, the accumulation and disappearance of membrane labilizing products of lipid hydrolysis on the other hand, are causally linked. The comparative studies on *E. coli* S_{15} and S_{17} indicate that the dose-dependent increase in sensitivity to actinomycin D, upon treatment with granulocyte fraction, is identical for the two strains, despite obvious differences in the extent of hydrolysis. This may reflect a dissociation of the two envelope effects. It is also possible that only a portion of the phospholipid degradation is responsible for the increase in permeability. Moreover, the action of the granulocyte phospholipase A_2 and of the *E. coli* phospholipase A_1 may well produce unequal functional effects, (1) because of the different products of hydrolysis formed, and (2) because hydrolysis may involve phospholipids belonging to different pools. For example, it is conceivable that the outer membrane associated phospholipase A_1 attacks primarily the inner layer of the phospholipid bilayer and that the granulocyte phospholipase A_2 first degrades phospholipids of the outer layer.

It is tempting to relate activation of the bacterial phospholipase and, consequently, greater phospholipid degradation to the greater bactericidal potency of the granulocyte fraction toward the parent strain. Comparable degradation and killing of the mutant strain is produced by higher concentrations of granulocyte fraction, consistent with such a relationship. It must be recognized, however, that differences may exist between the two strains that alter their sensitivities to this or other effects of the granulocyte fraction. Thus, *E. coli* S_{15} is more sensitive to the lytic effects of the detergent sodium dodecyl sulfate [24]. Such a difference in the membrane stability of the two strains could account for differences in their survival following exposure to the granulocyte fraction.

It is still unclear, therefore, whether phospholipid hydrolysis by granulocyte phospholipase or *E. coli* phospholipase is part of the bactericidal event or whether degradation merely represents a digestive process that accompanies and follows killing by granulocytes. The relationship of the granulocyte phospholipase A_2 to the other effects of the highly purified granulocyte fraction can only be more closely defined if, upon further purification, it can be established whether or not the phospholipase can be separated from the molecular species responsible for the effects on permeability and viability. Such attempts at continued dissection are in progress.

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