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Stimulation of Human Polymorphonuclear Leukocyte Oxidative Metabolism by Type 1 Pili from *Escherichia coli*

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We compared the degree to which *Escherichia coli* phase variants which do (T1P$^+$ *E. coli*) or do not (T1P$^-$ *E. coli*) express type 1 pili (T1P) stimulate human polymorphonuclear leukocyte (PMN) oxidative activity. Unopsonized T1P$^+$ *E. coli* stimulated the release of 0.20 to 0.24 nmol of H_2O_2 per 10^6 PMN per min and the consumption of 1.4 to 4.0 nmol of O_2 per 10^6 PMN per min; no measurable PMN oxidative activity was stimulated by unopsonized T1P$^-$ *E. coli*. In the presence of serum opsonins, T1P$^+$ *E. coli* stimulated the release of 1.12 to 1.16 nmol of H_2O_2 per 10^6 PMN per min and the consumption of 5.0 to 6.0 nmol of O_2 per 10^6 PMN per min, whereas T1P$^-$ *E. coli* stimulated the release of 0.42 to 0.43 nmol of H_2O_2 per 10^6 PMN per min and the consumption of 0.6 to 2.0 nmol of O_2 per 10^6 PMN per min. Although unaggregated T1P did not stimulate PMN, latex beads coated with T1P (T1P-latex) stimulated alpha-methylmannoside-inhibitable, opsonin-independent PMN oxidative activity. The activity stimulated by either T1P$^+$ *E. coli* or T1P-latex was susceptible to inhibition by cytochalasin B. Latex particles coated with bovine serum albumin or mannose-resistant pili did not stimulate PMN. These data indicate that T1P$^+$ *E. coli* stimulate PMN oxidative metabolism more effectively than do T1P$^-$ *E. coli* and that a similar PMN oxidative response follows cellular stimulation by either unopsonized T1P$^+$ or opsonized T1P$^-$ *E. coli*. Furthermore, T1P-latex faithfully mimics the ability of T1P$^+$ *E. coli* to stimulate PMN oxidative metabolism. Such particles may be useful in further analyses of cellular responses to T1P$^+$ *E. coli*.

Type 1 pili (T1P) are expressed by many virulent strains of *Escherichia coli* (20, 22, 25, 49). Such bacteria adhere to human buccal (31, 33) and urinary epithelial (22) cells and stimulate opsonin-independent polymorphonuclear leukocyte (PMN) oxidative, phagocytic, and secretory activity (4, 5, 27, 28, 35, 37, 42, 46, 47). However, such nonopsonic PMN activation induces less bacterial killing than does opsonic cellular activation (35, 45, 50; M. B. Goetz, S. M. Kuriyama, and F. J. Silverblatt, submitted for publication).

Since metabolically active PMN may harm the host (44, 51), we investigated the quantitative relationship between opsonic and nonopsonic PMN activation by phase variants of two strains of *E. coli* which do (T1P$^+$) and do not (T1P$^-$) express T1P. We also determined the ability of purified T1P to stimulate nonopsonic PMN oxidative metabolism and some of the requirements for the expression of such stimulation.

Previous studies have provided primarily qualitative rather than quantitative assessments of the ability of T1P$^+$ *E. coli* to stimulate opsonin-independent PMN oxidative metabolism (4, 5, 28, 35, 37, 42, 46, 47). Furthermore, although nonopsonic PMN oxidative metabolism is known to be stimulated by T1P$^+$ *E. coli*, T1P per se have not been shown to be an effective stimulus of such cellular activity (35, 42, 47).

MATERIALS AND METHODS

Chemicals. Phorbol myristate acetate was obtained from Consolidated Midland Corporation, Brewster, N.Y. Zymosan, cytochalasin B, concanavalin A (ConA), homovanillic acid, and horseradish peroxidase were from Sigma Chemical Co., St. Louis, Mo. Other reagents were of the highest available grade.

Bacterial strains. Other than for CSH50, all *E. coli* used in these experiments were derived from clinical isolates. Strains 2320, 346, and CSH50 express T1P after overnight growth in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) but not on blood or tryptic soy agar. Strain CSH50 expresses no pili other than T1P (16). *E. coli* 2320 also expresses pili which mediate mannose-resistant adherence to type A human erythrocytes at 4°C (MR pili) [17]. Strains 3025 and 4185 did not express T1P under any circumstances. The electron microscopic appearance and the ability of bacteria to agglutinate a suspension of *Candida albicans* in the presence of alpha-methylglucoside (aMG) but not alpha-methylmannoside (aMM) confirmed the presence of T1P (31, 32). Electron microscopic analyses were performed by fixing suspensions of bacteria or latex particles (vide infra) to copper grids which had been coated with Formvar and carbon. These grids were then washed, negative stained with 1% uranyl acetate, washed again, and examined with a Phillips 201 transmission electron microscope.

Preparation of PMN. PMN were routinely obtained by dextran sedimentation of heparinized venous blood obtained from healthy volunteers (7). Viability was always greater than 90% as determined by trypan blue exclusion. PMN were kept at 4°C in Dulbecco phosphate-buffered saline (PBS) supplemented with 2 mM KCN and 5 mM glucose until use.

Purification of pili. T1P were purified by the method of Eshdat et al. (19). Briefly, T1P were mechanically sheared from whole bacteria, precipitated in $MgCl_2$, and redissolved in 5 mM Tris, pH 8.0. This procedure was also used to isolate MR pili from *E. coli* 2320 grown on solid medium.

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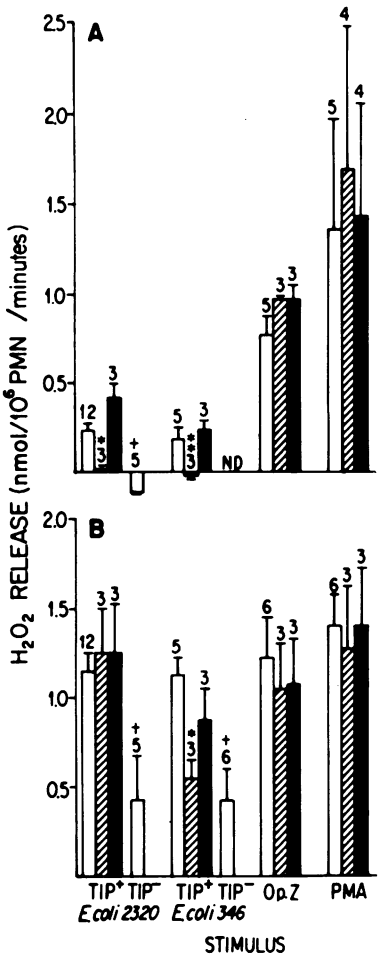

FIG. 1. Stimulation of PMN H_2O_2 release in the absence (A) or presence (B) of 10% fresh human serum. The bars depict the standard error of the mean (SEM), while the numbers above the bars represent the number of experiments. ND, Not done; Op.Z., opsonized zymosan. Open bars, Stimulation in the absence of aMM or aMG; cross-hatched bars, stimulation in the presence of 10 mM aMM; solid bars, stimulation in the presence of 10 mM aMG. Symbols: *, **, $P < 0.01$ or $P < 0.05$, respectively, compared with analogous sample run in the absence of aMM; +, $P < 0.01$ compared with analogous sample with the T1P$^+$ phase variant.

Protein concentrations were measured by the method of Lowry et al. (26).

Preparation of latex particles. Latex beads (0.81 μm; Difco Laboratories, Detroit, Mich.) were washed in 100 mM tricine buffer (pH 8.5) and then coincubated with 2.5 to 5.0 mg of T1P, MR pili, or bovine serum albumin (BSA) per ml in a final volume of 0.7 ml of the same buffer at 37°C for 30 min. All particles were washed in PBS before being used to stimulate PMN oxidative metabolism.

Assays of cellular oxidative metabolism. Phagocyte oxidative activity was assessed as a parameter of oxygen uptake and H_2O_2 release. The cells were stimulated by bacteria at a final ratio of 10 bacteria to 1 PMN, latex particles at a final ratio of 60 particles to 1 PMN, 1 mg of opsonized zymosan per ml; 100 μg of ConA per ml, or 1 μg of phorbol myristate acetate per ml. Zymosan was opsonized by coincubating one part of a 50-mg/ml solution of zymosan in PBS with two parts of fresh serum at 37°C for 30 min. The zymosan was then washed and resuspended in PBS before being used on the same day.

To assess PMN H_2O_2 release, we coincubated 2×10^6 PMN and the appropriate stimulus in sealed, tumbling tubes at 37°C with 0.2 mM homovanillic acid and 1.0 U of horseradish peroxidase in a final volume of 2 ml of PBS (43). After 60 min, the reaction was halted by the addition of 0.25 ml of ice-cold 25 mM EDTA–0.1 M glycine, pH 12. After centrifugation to remove particulate matter, the soluble oxidized homovanillic acid product was measured spectrofluorometrically with an excitation setting of 312 nm and an emission setting of 420 nm. Standard curves were run in both the presence and absence of serum. All test conditions were done in duplicate daily. In preliminary studies, we demonstrated that the amount of the fluorescence bore a linear relationship to the amount of H_2O_2 present with a lower limit of 10 nmol in the presence of fresh serum and 2 nmol in the absence of serum. Under both conditions the upper limit was over 200 nmol. No H_2O_2 was detected if PMN were deleted from the assay. The specificity of the assay for H_2O_2 was confirmed by the ability of fresh, but not boiled, catalase to inhibit the conversion of homovanillic acid to the fluorescent product.

O_2 uptake was measured polarographically with an Oxygraph (Gilson Medical Electronics, Middleton, Wis.) outfitted with a Clark oxygen electrode and an airtight, temperature-controlled, water-jacketted cuvette (38). PMN (8×10^6) were preincubated at 37°C in the 1.6-ml assay chamber for 5 min before being stimulated. Oxygen uptake was linear from 1 to 5 min after PMN stimulation. The oxygen content of PBS was assessed by the method of Robinson and Cooper (40). All conditions including control assays in which PMN were not stimulated were done in duplicate daily. The oxygen consumed by the control PMN was subtracted from that of the other samples before any calculations were done.

Statistics. Student's two-tailed *t* test for paired or unpaired samples were used as appropriate for all statistical analyses. The mean of duplicate assays done in a single day was treated as being the results of a single experiment.

RESULTS

T1P$^+$ *E. coli*-mediated stimulation of PMN oxidative metabolism. T1P$^+$ phase variants of *E. coli* 2320 and 346, but not T1P$^-$ phase variants of *E. coli* 2320, stimulated nonopsonic PMN H_2O_2 release (Fig. 1A). As with other T1P-mediated phenomena (3, 5, 42, 45), the ability of T1P$^+$ *E. coli* to stimulate nonopsonic cellular H_2O_2 release was completely ablated by 10 mM aMM but not by 10 mM aMG. Neither aMM nor aMG affected PMN H_2O_2 release after stimulation with phorbol myristate acetate or opsonized zymosan. In the presence of serum opsonins, both T1P$^+$ and T1P$^-$ phase variants of *E. coli* 2320 and 346 stimulated PMN H_2O_2 release; such stimulation was not consistently inhibited by aMM (Fig. 1B). The constitutively T1P$^-$ *E. coli* 3025 or 4185 did not stimulate nonopsonic cellular H_2O_2 release regardless of whether the bacteria were grown on solid or in liquid media (data not shown). Thus, alterations in bacterial surface properties, e.g., endotoxin content or hydrophobicity, owing to growth in liquid rather than on solid media are not sufficient to stimulate nonopsonic PMN H_2O_2 release.

Since measurement of H_2O_2 assesses only the release or extracellular leakage of oxidative products and not necessarily the total magnitude of intracellular oxidative activity (11, 41), we determined the rate of PMN O_2 uptake after stimulation of T1P$^+$ or T1P$^-$ *E. coli* (Fig. 2). The relative

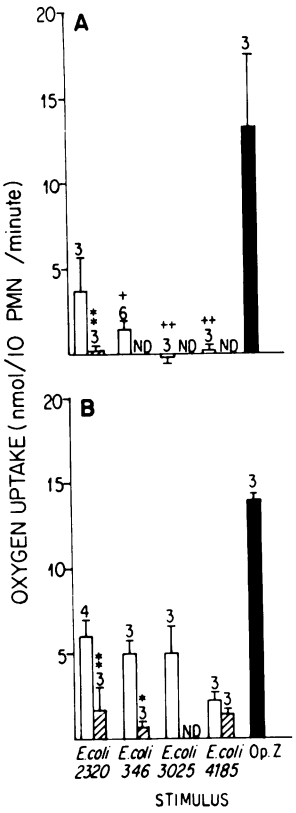

FIG. 2. Stimulation of PMN O_2 consumption in the absence (A) or presence (B) of 10% fresh human serum. The bars depict the SEM, while the numbers above the bars represent the number of experiments. ND, Not done; Op.Z., opsonized zymosan. Open bars, Stimulation by bacteria grown in liquid media; cross-hatched bars, stimulation by bacteria grown on solid media. *E. coli* 2320 and 346 but not *E. coli* 3025 and 4185 express T1P when grown in liquid media. Symbols: *, **, $P < 0.01$ or $P < 0.05$, respectively, compared with analogous sample with bacteria grown in broth; +, ++, $P < 0.01$ or $P < 0.05$, respectively, compared with analogous sample run in the presence of serum.

magnitude of O_2 uptake correlated with the amount of PMN H_2O_2 release although the precise ratio of cellular H_2O_2 release to O_2 uptake varied from one condition to another. Thus, variable sealing of phagolysosomes (9) does not account for the greater stimulation of opsonic PMN H_2O_2 release by $T1P^+$ *E. coli* than by $T1P^-$ *E. coli*.

Stimulation of PMN oxidative activity by purified T1P. To determine whether T1P per se stimulate nonopsonic PMN oxidative metabolism, we challenged the cells with latex particles coated with purified T1P isolated from *E. coli* 2320, 346, or CSH50 (T1P-latex). These latex particles agglutinated *C. albicans* in the presence of 10 mM aMG but not 10 mM aMM. As controls, latex particles were coated with either BSA (BSA-latex) or with MR pili from $T1P^-$ *E. coli* 2320 (MR pili-latex). Such particles did not agglutinate *C. albicans*. Electron microscopy demonstrated that the orientation of T1P or MR pili on latex particles was similar to that of T1P on *E. coli* (Fig. 3). (T1P-latex) but not BSA-latex adhered to PMN monolayers in the absence of serum (data not shown).

T1P-latex but not BSA-latex stimulated nonopsonic PMNL H_2O_2 release (Fig. 4). In the presence of serum opsonins both T1P-latex and BSA-latex stimulated cellular H_2O_2 release. Furthermore, T1P-latex but not BSA-latex or MR pili-latex stimulated PMN O_2 consumption (Fig. 5). O_2 consumption commenced within 1 min of PMN stimulation by latex particles coated with T1P purified from *E. coli* 2320, 346, or CSH50.

As has been previously shown (28), unaggregated suspensions of purified T1P at 0.05 to 0.10 mg of protein per ml did not stimulate either assay of PMN oxidative activity regardless of the presence of serum opsonins. If it is assumed that when the T1P-latex particles are prepared all pili become adherent to the latex beads, the final concentration of T1P was no more than 0.04 mg/ml in assays of H_2O_2 release and 0.2 mg/ml in assays of O_2 uptake.

The T1P preparations used in these studies are likely to be contaminated by endotoxin. Thus, either direct activation or priming of PMN oxidative metabolism by endotoxin might have accounted for the stimulatory activity of T1P (13, 15, 21, 23; R. L. Danner, K. A. Joiner, M. Rubin, J. W. Hathorn, R. L. Schanfele, and J. E. Parrillo, Clin. Res.

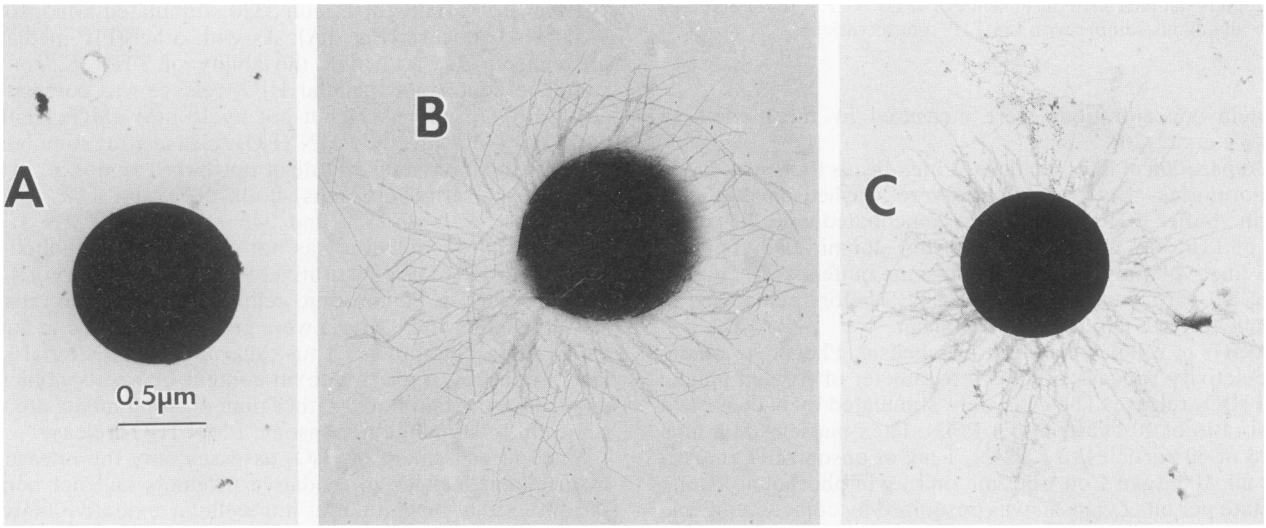

FIG. 3. (A) Photomicrograph of a latex particle which has been coated with BSA. (B) Particle coated with T1P. (C) Particle coated with MR pili.

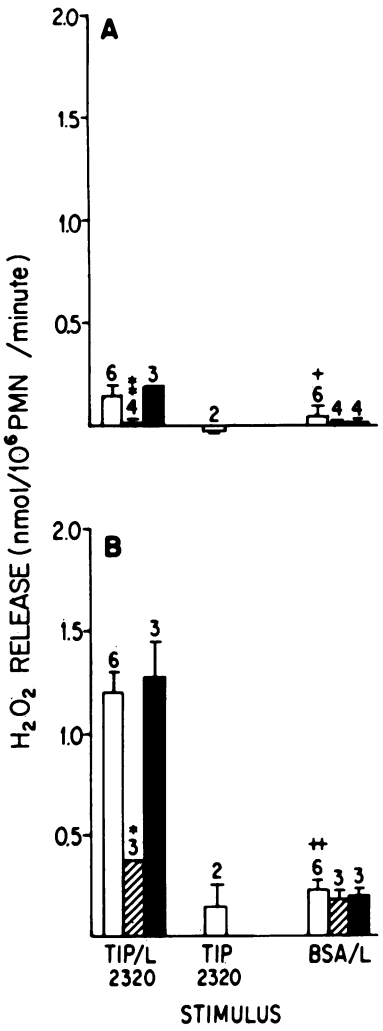

FIG. 4. Stimulation of PMN H_2O_2 release in the absence (A) or presence (B) of 10% fresh human serum. The bars depict the SEM, while the numbers above the bars represent the number of experiments. ND, Not done; T1P/L, T1P-latex; BSA/L, BSA-latex; T1P 2320, free purified T1P from *E. coli* 2320. Open bars, Stimulation in the absence of aMM or aMG; cross-hatched bars, stimulation in the presence of 10 mM aMM; solid bars, stimulation in the presence of 10 mM aMG. Symbols: *,**, $P < 0.01$ or $P < 0.05$, respectively, compared with analogous sample run in the absence of aMM; +, ++, $P < 0.01$ or $P < 0.05$, respectively, compared with analogous sample with T1P-latex 2320.

33:764A, 1985). These properties of endotoxin are inhibited by polymyxin B (2, 12, 23; Danner et al., Clin. Res, 1985). We therefore determined whether PMN preincubation for 15 min in the presence or absence of 10 μg of polymyxin B per ml altered T1P-latex-mediated cellular stimulation. In the absence of polymyxin B, *E. coli* T1P-stimulated PMN consumed 8.44 ± 1.25 nmol of O_2 per min per 5×10^6 cells, whereas in the presence of polymyxin B such PMN consumed 8.39 ± 0.99 nmol of O_2 per min per 5×10^6 cells ($n = 5$, $P > 0.05$).

These data demonstrate that, as with $T1P^+$ *E. coli*, aMM but not aMG inhibited T1P-latex-mediated stimulation of PMN H_2O_2 release and O_2 uptake. Moreover, opsonized T1P-latex stimulated O_2 uptake and H_2O_2 release much more effectively than did unopsonized T1P-latex.

Requirements for nonopsonic PMN stimulation by T1P. aMM inhibits the attachment of both T1P and ConA to PMN (5, 27, 42, 45). We compared the effect of cytochalasin B on cellular stimulation by these agonists to determine whether this homology extends to the means by which these agents activate PMN oxidative metabolism.

PMN preincubation with cytochalasin B for 10 min before being stimulated did not alter the adherence of $T1P^+$ *E. coli* or of T1P-latex to the phagocytes (data not shown). In contrast, cytochalasin B strongly inhibited stimulation of nonopsonic and opsonic PMN H_2O_2 release by $T1P^+$ *E. coli* T1P-latex, but not by ConA (Fig. 6).

DISCUSSION

These data demonstrate that when compared with opsonized $T1P^+$ *E. coli*, unopsonized $T1P^+$ *E. coli* and opsonized $T1P^-$ *E. coli* are weaker but essentially equipotent stimuli of PMN oxidative metabolism. Although opsonized $T1P^-$ *E. coli* stimulated somewhat more cellular H_2O_2 release than did unopsonized $T1P^+$ *E. coli*, the converse was true when cellular O_2 uptake was examined. The absolute rates of H_2O_2 release and O_2 uptake are not directly comparable since the former assay measured cellular activity over 1 h, while the latter technique assessed oxidative activity over 5 min. PMN oxidative activity generally begins to subside 15 min after cellular stimulation (1). Furthermore, whereas O_2 uptake measures total PMN oxidative metabolism, H_2O_2 release reflects only the extracellular release of oxidative products (11, 41).

The observed magnitude of PMN oxidative activity does not correlate with the rate at which PMN kill *E. coli* 2320. Although opsonized $T1P^+$ *E. coli* constituted the most effective stimulant of cellular oxidative activity, opsonized

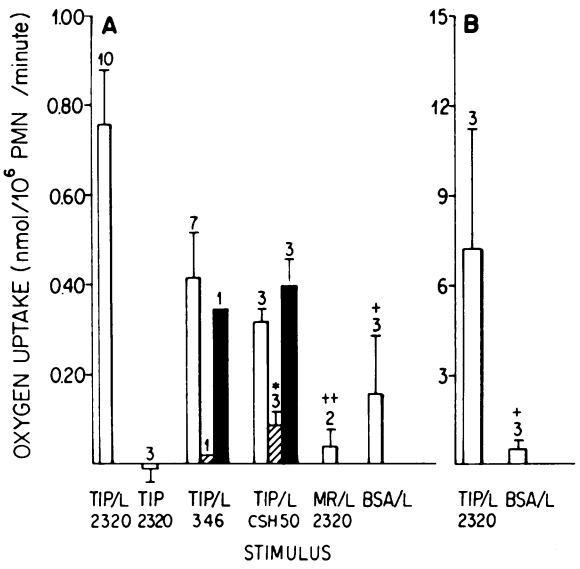

FIG. 5. Stimulation of PMN O_2 consumption in the absence (A) or presence (B) of 10% fresh human serum. The bars depict the SEM, while the numbers above the bars represent the number of experiments. ND, Not done; T1P/L, T1P-latex; BSA/L, BSA-latex; T1P 2320, free purified T1P from *E. coli* 2320. Open bars, Stimulation in the absence of aMM or aMG; cross-hatched bars, stimulation in the presence of 10 mM aMM; solid bars, stimulation in the presence of 10 mM aMG. Symbols: *, $P < 0.01$ compared with analogous sample run in the absence of aMM; +, ++, $P < 0.01$ or $P < 0.05$, respectively, compared with analogous sample with T1P-latex 2320.

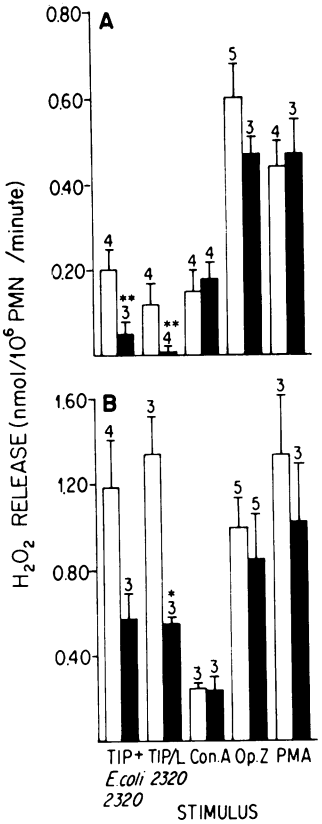

FIG. 6. Stimulation of PMN H_2O_2 release in the absence (A) or presence (B) of 10% fresh human serum. The bars depict the SEM, while the numbers above the bars represent the number of experiments. Open bars, Stimulation in the absence of cytochalasin B; closed bars, stimulation in the presence of 2.5 μg of cytochalasin B per ml. Symbols: *, **, $P < 0.01$ or $P < 0.05$, respectively, compared with analogous sample run in the absence of cytochalasin B.

T1P$^+$ and T1P$^-$ *E. coli* 2320 were are equally susceptible to PMN bactericidal activity, whereas unopsonized T1P$^+$ *E. coli* are, at most, killed only 15 to 20% as quickly (Goetz et al., submitted). These data imply that unopsonized T1P$^+$ *E. coli* 2320 cells are intrinsically more resistant to oxygen-dependent PMN bactericidal mechanisms than are opsonized T1P$^-$ *E. coli* cells or that oxidative products or other PMN bactericidal products do not accumulate in the vicinity of such bacteria. We have shown that less myeloperoxidase activity is found within phagolysosomes which contain unopsonized T1P$^+$ *E. coli* than within phagolysosomes which contain opsonized T1P$^+$ or T1P$^-$ *E. coli* (Goetz et al., submitted).

Previously, T1P have been considered to be an agonist of PMN oxidative activity solely because T1P$^+$ *E. coli* but not T1P$^-$ *E. coli* stimulate aMM-inhibitable PMN oxidative metabolism (5, 35, 42, 47). Such indirect evidence may be subject to confounding factors. For example, varying the growth media or otherwise treating bacteria to select for or against the expression of T1P may also affect the expression of other bacterial surface attributes which in turn stimulate PMN oxidative metabolism.

We used two means to confirm that T1P per se stimulate PMN oxidative metabolism. First, we determined that after growth in a medium which promotes T1P expression, two strains of *E. coli* which are constitutively T1P$^-$ did not stimulate nonopsonic PMN oxidative metabolism. We also demonstrated that latex particles coated with T1P from T1P$^+$ *E. coli* 2320, 346, or CSH50 stimulated aMM-inhibitable, nonopsonic PMNL H_2O_2 release and O_2 uptake. As was observed with T1P$^+$ *E. coli*, opsonization markedly increased the ability of T1P-latex to stimulate PMN. Latex particles coated with BSA or with MR pili purified from T1P$^-$ *E. coli* 2320 did not effectively stimulate nonopsonic PMNL H_2O_2 release and O_2 uptake. The disparity between the magnitude of cellular oxidative activity after PMN stimulation by latex particles coated with T1P isolated from from *E. coli* 2320, CSH50, and 346 may reflect intrinsic heterogeneity of T1P (24, 39). Increasing the concentration of CSH50 or 346 T1P on latex particles did not promote further stimulation of PMN O_2 uptake (data not shown).

These data indicate that T1P per se mediate stimulation of PMN oxidative metabolism. It is highly unlikely that a non-T1P contaminant, i.e., endotoxin, is responsible for the stimulation of PMN oxidative metabolism observed after phagocyte exposure to T1P-latex. First, although endotoxin may contaminate our preparations of T1P, the susceptibility of T1P-latex stimulation to inhibition by aMM has not been described with endotoxin. Second, stimulation of PMN oxidative metabolism by T1P-latex is not inhibited by polymyxin B although polymyxin B inhibits such PMN activation by endotoxin (2, 12, 23; Danner et al., Clin. Res., 1985). Third, direct stimulation or potentiation of PMN oxidative activity by endotoxin requires a 20- to 40-min period during which the cells become adherent to a surface (8, 13–15, 21, 52), whereas the stimulation of PMN O_2 uptake after exposure to T1P-latex occurs within 1 min. Finally, endotoxin would also be expected to contaminate preparations of unaggregated T1P and MR pili, yet neither of these materials stimulated PMN oxidative metabolism. The failure of MR pili-latex to stimulate PMN also implies that other bacterial products which are PMN agonists, such as formyl-methionyl-leucyl-phenylalanine (6, 29), are not significant contaminants of our preparations of T1P.

These studies also establish some of the necessary conditions which must be met for T1P to stimulate PMN. We demonstrated that whereas free T1P per se (28) are not effective agonists of PMN oxidative metabolism, latex particles coated with T1P do activate phagocytes. These data support the concept that spatial constraints may determine the responsiveness of PMN oxidative metabolism to stimulation by T1P. Perry et al. (37) have reported that T1P$^+$ *E. coli* more effectively stimulate PMN oxidative metabolism when the pili are first cross-linked by either antibody or glutaraldehyde. Furthermore, since cytochalasin B inhibited PMN activation by T1P$^+$ *E. coli* or T1P-latex without impairing their attachment to PMN, stimulation by T1P is likely to require intact PMN microfilament function. Thus, although cellular stimulation by both T1P and ConA is subject to inhibition by aMM (3, 10, 18, 46), our studies indicate that T1P and ConA stimulate PMN oxidative activity by dissimilar means since ConA-mediated stimulation is promoted rather than inhibited by cytochalasin B.

In summary, these data demonstrate that a quantitative defect of cellular oxidative activity does not account for the poor killing of unopsonized T1P$^+$ *E. coli*, that inert particles coated with T1P, but not free T1P per se, faithfully reproduce the ability of T1P$^+$ *E. coli* to stimulate both opsonin-dependent and opsonin-independent cellular oxidative metabolism, and that stimulation of PMN by T1P-coated particles is susceptible to inhibition by cytochalasin B. The properties of T1P-latex provide a new and useful tool with

which to analyze PMN responses to T1P and to determine what structural and functional aspects of T1P stimulate PMN (19, 34, 36). Such studies are of particular interest since recent data indicate that T1P adherence to guinea pig erythrocytes may be mediated by a minor pilus component rather than by the major structural subunit (30).

Finally, our findings suggest another means by which T1P may enhance the virulence of *E. coli*. Since the expression of T1P dramatically increases *E. coli*-mediated stimulation of PMN oxidative metabolism without substantially increasing bacterial susceptibility to PMN-mediated killing regardless of the presence of serum opsonins (Goetz et al., submitted), the accompanying inflammatory response may be detrimental to the host (44, 48, 51).

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