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Endotoxin-Induced Selective Dysfunction of Rabbit Polymorphonuclear Leukocytes in Response to Endogenous Chemotactic Factors

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To assess the mechanism and specificity of polymorphonuclear leukocyte (PMN) dysfunction induced by endotoxin, rabbits were injected intravenously with 100 μg of *Escherichia coli* endotoxin, and PMN function was studied 18 to 24 h later. Compared to PMN from normal rabbits, peripheral blood PMN from rabbits injected with endotoxin showed diminished chemotactic responsiveness to two endogenous peptides, C5a (complement) and platelet-derived growth factor, and to two endogenous lipids, leukotriene B_4 and platelet-activating factor. The chemotactic response to the synthetic chemotactic peptide, *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), was unimpaired. In contrast to migration, endotoxin injection resulted in inhibition of the secretory response to the two endogenous peptides but not to the lipids or to FMLP. At a 1:4 (vol/vol) dilution, the plasma either 1 or 24 h after the endotoxin injection inhibited normal PMN chemotactic responses to C5a but not to FMLP. Similarly, at a 1:10 dilution, this plasma inhibited normal PMN chemotactic responses to leukotriene B_4. The factor responsible for inhibiting responses to leukotriene B_4 was anionic, specific for leukotriene B_4 responses, and greater than 12,000 daltons. These data may be relevant to understanding PMN dysfunction during gram-negative sepsis.

Injection of endotoxin (lipopolysaccharide [LPS]) alters the migration of polymorphonuclear leukocytes (PMN). Verghese and Snyderman (28) have reported that endotoxin depresses the accumulation of PMN in the peritoneal cavity of mice in response to different stimuli. We have shown that intravenously injected endotoxin inhibits the PMN migration and capillary permeability induced in rabbit skin by a complement-mediated inflammatory reaction (22). We also showed that PMN which were obtained from rabbits 24 h after injection of intravenous endotoxin exhibited diminished chemotaxis and release of lysosomal enzymes in response to C5a (22).

PMN migration to a site of infection or inflammation is theoretically attributable to several endogenous chemotactic substances released as a consequence of the inflammatory process. Important mediators responsible for PMN migration include the complement-derived peptide C5a, the arachidonic acid metabolite leukotriene B_4 (LTB_4), and platelet-activating factor (PAF) (6, 8, 20). These substances, in addition to being chemotactic, also induce the release of lysosomal enzymes from PMN (8, 20, 25). To characterize further the specificity and mechanism of PMN dysfunction induced by endotoxin, we have used a variety of neutrophil stimuli to study chemotaxis and release of beta-glucuronidase from PMN obtained from rabbits injected with endotoxin.

MATERIALS AND METHODS

Animals. Female New Zealand White rabbits (Animals West, Santa Cruz, Calif.) weighing approximately 2 kg were housed in the animal care facilities of either San Francisco General Hospital or the Medical Research Institute of San Francisco. Animals were fed standard laboratory chow.

Endotoxin. LPS prepared by the Westphal extraction of *Escherichia coli* O55:B5 was obtained from Difco Laboratories, Detroit, Mich. (lot no. 682197; 6.08% lipid A). LPS was suspended in sterile, pyrogen-free saline and stored at −20°C.

Isolation of PMN. Rabbit PMN were isolated from acid-citrate-glucose-anticoagulated blood obtained from the central ear artery. Thirty-four milliliters of blood plus 6 to 8 ml of anticoagulant were mixed with 8 ml of 6% (wt/vol) Dextran T-500 (Pharmacia, Inc., Piscataway, N.J.) in normal saline. After sedimenting for approximately 30 min, the supernatant was layered over 56% Percoll (Sigma Chemical Co., St. Louis, Mo.) and centrifuged at 450 × *g* for 20 min. The erythrocyte-PMN pellet from this centrifugation was suspended in 40 ml of 8.3% (wt/vol) ammonium chloride (pH 7.2) to produce erythrocyte lysis. After 7 min, PMN were centrifuged for 10 min at 150 × *g* and washed twice in phosphate-buffered saline. We have found that this method for rabbit PMN isolation produces greater than 95% viability as judged by trypan blue exclusion and greater than 95% homogeneity as judged by light microscopic appearance.

Chemotaxis. For chemotactic studies, PMN were suspended in Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) plus 2% bovine serum albumin (Sigma) at a concentration of 2.5×10^6/ml. Migration was assayed by using modified Boyden chambers. Filters were stained after incubation for 35 min at 37°C. Results were analyzed by the leading front approach as described by Zigmond and Hirsch (31). Net migration was calculated as the movement in response to a stimulus minus the random migration (movement in response to buffer alone). All stimuli were tested in duplicate and results represent readings from

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TABLE 1. PMN from LPS-treated rabbits show a diminished chemotactic response to many but not all stimuli

Stimulus	Concn	Net migration (μm/35 min) of PMN from[a]		P[b]
		Normal rabbits	LPS-treated rabbits	
C5a	0.1%	72.0 ± 4.0 (6)	21.3 ± 5.1 (6)	<0.001
PDGF	1 U/ml	44.8 ± 10.2 (5)	5.4 ± 5.5 (5)	<0.005
LTB_4	5 ng/ml	61.0 ± 6.8 (5)	8.8 ± 2.4 (5)	<0.005
PAF	0.1 μg/ml	12.6 ± 3.4 (5)	0.1 ± 4.2 (5)	<0.025
FMLP	10^{-9} M	35.0 ± 2.4 (6)	36.0 ± 5.4 (6)	NS

[a] Net migration represents the average migration in response to a stimulus minus the migration in response to buffer alone. Numbers in parentheses represent the number of animals studied. Values are expressed as the mean ± standard error.
[b] *P* values are derived from Student's *t* test. NS, Not significant.

a minimum of five fields per nitrocellulose filter (pore diameter, 3 μm; Sartorius, San Francisco, Calif.).

Enzyme release. To quantitate the release of the azurophilic granule enzyme beta-glucuronidase, PMN were suspended in Hanks balanced salt solution plus 2% bovine serum albumin at a concentration of 4.5×10^6 to 5×10^6 PMN per ml. PMN were incubated with cytochalasin B (5 μg/ml; Aldrich Chemical Co., Inc., Milwaukee, Wis.) (5 μg/ml) for 5 min at 37°C. PMN were then exposed to appropriate stimuli for an additional 15 min at 37°C. Cell-free supernatants were obtained by centrifuging the PMN suspensions for 5 min at 900 × *g*. Total enzyme activity was determined from lysates of cells exposed to 0.3% (vol/vol) Triton X-100, the detergent. Background enzyme activity in the supernatant was determined by centrifugation of cells immediately after the cytochalasin B exposure. Nonspecific enzyme release was calculated based on the enzyme present in supernatants of cells incubated for 15 min at 37°C without a specific stimulus. All stimuli were tested in duplicate. Glucuronidase activity was measured as described previously, with phenolphthalein glucuronide (Sigma) as a substrate (11, 22). Release of the cytoplasmic enzyme, lactate dehydrogenase, was assayed by the method of Wacker et al. and used as an index of cell viability (29).

Chemotactic factors. Chemotactic factors included: *N*-formyl-methionyl-leucyl-phenylalanine (FMLP; Peninsula Labs, San Carlos, Calif.), LTB_4 (the generous gift of Joshua Rokach, Merck, Frosst, Canada), PAF (Bachem, Torrance, Calif.), platelet-derived growth factor (PDGF; Collaborative Research, Waltham, Mass.), and rabbit C5a, partially purified as previously described (22). As described for highly purified PDGF (4), the chemotactic activity present in this commercial preparation was markedly inhibited by protamine (20 μg/ml), which did not affect C5a as a chemoattractant. The C5a preparation induced an optimal chemotactic response at a protein concentration of 12 μg/ml as determined by the Lowry method. The two lipid mediators, PAF and LTB_4, were stored in methanol at −20°C while the peptide stimuli, FMLP, C5a, and PDGF, were stored in water or saline at −20°C and diluted appropriately just before use. The methanol from the lipid mediators was evaporated under nitrogen, and the stimuli were suspended in buffer containing bovine serum albumin immediately before their use. Dimethylsulfoxide was used to solubilize the FMLP but was present at a concentration of less than 0.001% in the chemotaxis assays.

Plasma incubations. PMN from normal rabbits were incubated for 20 min at 37°C with normal EDTA-anticoagulated plasma or plasma either 1 or 24 h after intravenous injection of 100 μg of *E. coli* LPS. In the majority of studies, the rabbit was bled before the LPS injection, and its own normal plasma was used as a control. For the incubations, PMN were suspended at a concentration of 10^7/ml. Plasma incubations were performed at both a 10 and 25% concentration (vol/vol) relative to the total volume of cells plus buffer. In most of the studies in which a 10% plasma concentration was used, plasma was dialyzed against phosphate-buffered saline before testing by using dialysis tubing with a molecular weight cutoff of 12,000. Dialysis was used as an initial step in estimating the molecular weights of any inhibiting factors. After plasma incubation, PMN were washed, suspended in buffer, and tested for chemotactic responsiveness to buffer, FMLP, C5a, or LTB_4 in optimal or nearly optimal concentrations.

Ion exchange column chromatography. EDTA-anticoagulated plasma was dialyzed against 10 mM phosphate buffer (pH 7.5) by using tubing with a molecular weight cutoff of 12,000. The plasma was loaded onto a DEAE-cellulose (Bio-Rad Laboratories, Richmond, Calif.) ion exchange column, and eluates were monitored for A_{280}. The column was eluted serially with 10 mM phosphate buffer (pH 7.5), with a pH and salt gradient to pH 6.5, with 300 mM phosphate, and finally with 2 M NaCl (pH 6.3). Eluates were dialyzed against phosphate-buffered saline (pH 7.2). Column fractions were incubated with normal rabbit PMN as in the plasma studies noted above. The inhibitory activity of the column fraction is expressed relative to the migration of PMN incubated with phosphate-buffered saline.

Statistics. Statistical comparisons are based on Student's *t* test.

RESULTS

In preliminary studies, dose response curves were derived for PMN from normal rabbits with each chemotactic stimulus. Since PDGF has not previously been reported as a rabbit PMN chemoattractant, a checkerboard analysis (30) was used to confirm that it was chemotactic and not merely chemokinetic. An optimal or suboptimal concentration for each stimulus was then selected for further testing by using both PMN from normal rabbits and PMN from rabbits that had been injected with 100 μg of *E. coli* LPS intravenously 18 to 24 h previously. Intravenous injection of LPS did not affect PMN responses to FMLP (Table 1). In contrast, PMN chemotactic responses to all other stimuli tested were significantly diminished. These stimuli included two peptides, C5a and PDGF, and two lipids, LTB_4 and PAF. Intravenous LPS somewhat enhanced the migration in response to buffer alone (82 ± 14 μm/35 min for PMN from LPS-injected rabbits versus 66 ± 5 μm/35 min for PMN from control rabbits; $P < 0.05$). PMN from LPS-injected rabbits responded comparably to PMN from normal rabbits with reference to C5a, FMLP, and LTB_4 when PMN were tested 48 h after the LPS injection.

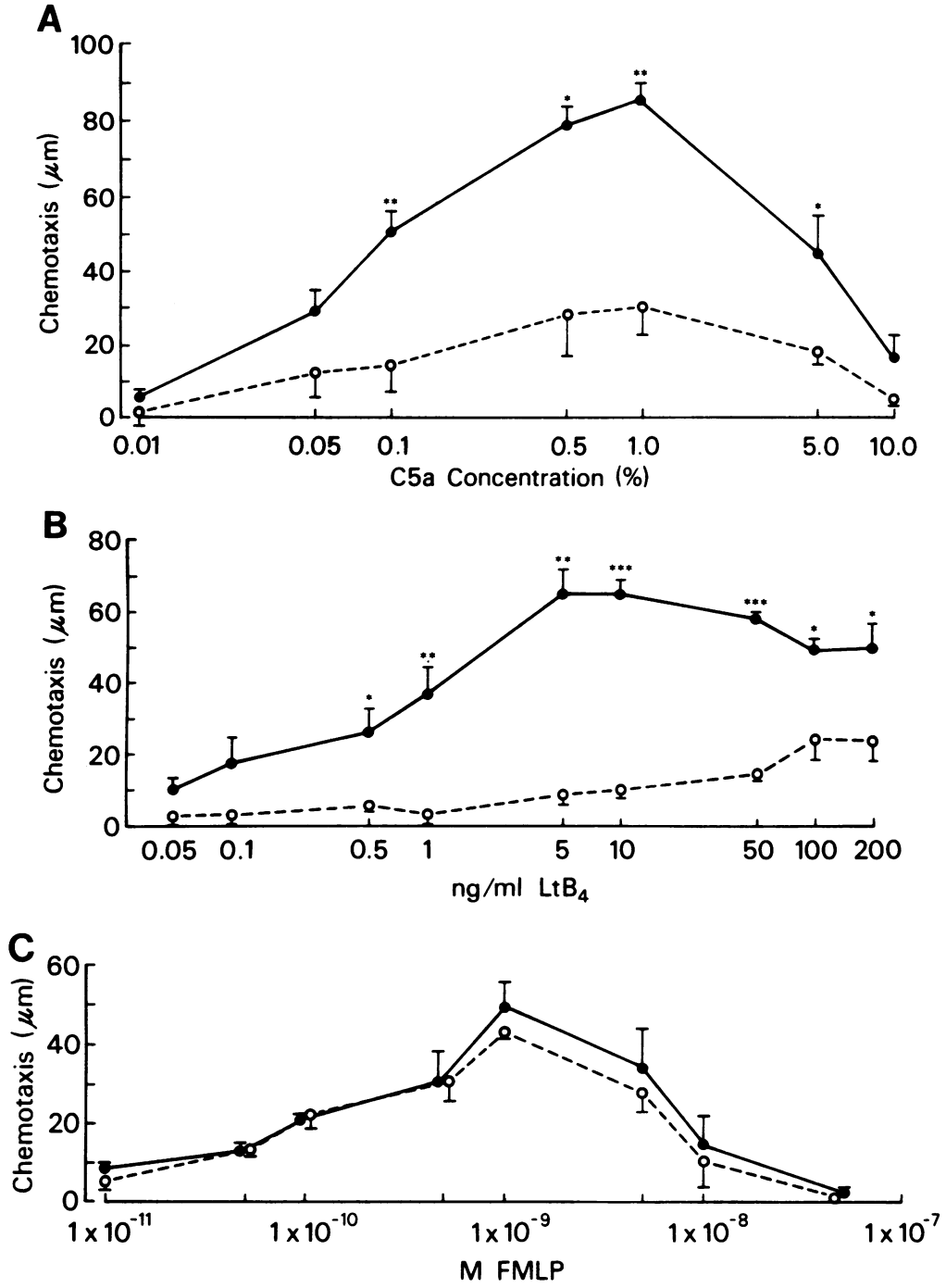

FIG. 1. Chemotaxis dose response curves for PMN from normal rabbits (●) (n = 3) and PMN from rabbits 24 h after LPS (○) (n = 3) for partially purified rabbit C5a (A) LTB_4 (B), and FMLP (C). Statistical comparisons are based on a Student's t test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

To consider the possibility that diminished chemotactic responsiveness might merely represent a shift in the dose response curve, chemotactic responsiveness for three of the mediators was tested over a range of concentrations. The chemotactic responses of PMN from LPS-treated rabbits for LTB_4 and C5a were diminished over a wide range of concentrations, while FMLP responses were not affected for concentrations ranging from 5×10^{-11} to 1×10^{-8} M (Fig. 1).

In addition to migration, PMN are capable of releasing their granule contents in response to chemotactic stimuli. PMN from LPS-treated rabbits released the primary granule enzyme, beta-glucuronidase, in response to FMLP comparably to PMN from normal rabbits (Table 2). The secretory response to the two peptide stimuli, C5a and PDGF, was significantly reduced by a prior intravenous endotoxin injection. For the two lipid stimuli, LTB_4 and PAF, intravenous

TABLE 2. PMN from LPS-treated rabbits show a diminished secretory response to C5a and PDGF but not FMLP, LTB, or PAF

Stimulus	Beta-glucuronidase release (% total enzyme)[a]		P[b]
	Normal PMN	Post-LPS PMN	
C5a (1%)	31.8 ± 4.9 (7)	17.7 ± 4.4 (7)	<0.025
PDGF (4.5 to 5 U/ml)	3.8 ± 1.1 (5)	1.6 ± 0.9 (5)	<0.025
FMLP (10^{-10}M)	4.6 ± 1.5 (4)	3.9 ± 2.0 (4)	NS
FMLP (10^{-9}M)	27.1 ± 6.0 (7)	32.6 ± 7.3 (7)	NS
FMLP (10^{-8}M)	49.9 ± 4.9 (5)	57.8 ± 6.4 (5)	NS
LTB_4 (25 ng/ml)	1.5 ± 0.6 (5)	3.1 ± 1.4 (5)	NS
LTB_4 (100 ng/ml)	5.4 ± 1.7 (5)	4.0 ± 1.2 (5)	NS
PAF (1 μg/ml)	16.1 ± 2.8 (7)	16.1 ± 3.1 (7)	NS

[a] Numbers in parentheses give the number of animals studied. Values are the mean ± standard error for each variable studied. Values represent the enzyme release in the supernatant of stimulated cells minus the enzyme released spontaneously in unstimulated cells that had also been incubated at 37°C. This nonspecific enzyme release was always less than 5% of the total enzyme present in detergent-lysed cells.

[b] P values are derived from Student's t test. NS, not significant.

administration of LPS did not produce a significant change in the secretory responsiveness. This contrasts with the chemotactic responsiveness which was diminished for all stimuli tested except FMLP. None of the stimuli tested caused significant release of the cytoplasmic enzyme, lactate dehydrogenase.

Incubation of PMN with 25% plasma obtained either 1 or 24 h after intravenous LPS significantly reduced PMN responses to C5a without significantly reducing responses to FMLP (Table 3). With 25% plasma, LTB_4 responses were significantly decreased only by incubation with the plasma 1 h after treatment with LPS. Incubating PMN with 10% plasma either 1 or 24 h after treatment with LPS consistently inhibited the LTB_4 response without significantly diminishing the response to C5a ($P < 0.005$). At a 10% concentration, plasma from rabbits that received LPS 24 h (but not 1 h) previously slightly inhibited responsiveness to FMLP ($0.05 > P > 0.025$). None of the concentrations of plasma tested at either time affected migration in response to buffer alone.

To characterize further the inhibitor of responses to LTB_4, plasma 1 h after intravenous injection of endotoxin was dialyzed against a 10 mM phosphate buffer and then chromatographed on a DEAE-cellulose column. Anionic fractions of plasma that eluted just after the albumin peak reduced the responsiveness of normal PMN to LTB_4 (Fig. 2). To determine the specificity and reproducibility of this observation, the assay was repeated with plasma from six different collections. Anionic substances which elute just after albumin have consistently reduced the response of normal PMN to LTB_4 (average reduction, 21.9 ± 4.7 μm/35 min; $n = 13$; $P < 0.005$) without significantly affecting responses to C5a (average inhibition, 4.9 ± 3.5 μm/35 min; $n = 10$), to FMLP (average inhibition, 0.3 ± 4.3 μm/35 min; $n = 7$), or to buffer alone (average increase, 0.2 ± 1.5 μm/35 min; $n = 13$).

DISCUSSION

In the present study, we evaluated the effect of intravenous endotoxin on the function of rabbit PMN. Our studies indicate that a single injection of endotoxin results in a transient inability of PMN to migrate toward all tested endogenous chemotactic factors, whereas their ability to migrate towards the synthetic chemotactic peptide FMLP is not affected. Injection of endotoxin slightly enhanced random migration, a finding not noted in prior studies (22). In recent unpublished studies, highly purified endotoxin preparations (Ribi Immunochem, Hamilton, Mont.) have induced stimulus-specific PMN dysfunction without consistently increasing random migration. The ability of PMN to release granule contents is also reduced by an intravenous injection of endotoxin, but only in response to the two endogenous peptide stimuli, C5a and PDGF, and not in response to the lipids, LTB_4 and PAF, or the exogenous peptide, FMLP. The endotoxin-induced inhibition of PMN chemotactic responses was partially transferable by plasma which inhibited normal PMN chemotaxis to LTB_4 and C5a but not usually to FMLP. Results of ion exchange chromatography and dialysis suggested that the factor responsible for inhibiting the responsiveness to LTB_4 is anionic, with a molecular weight greater than 12,000.

These findings may be relevant to gram-negative sepsis which may be associated with endotoxemia (13). Although differing in techniques and conclusions, several reports have indicated altered PMN function during gram-negative infection (1, 2, 5, 12, 14, 16, 18, 27).

We reported previously that endotoxin results in an inhibition of the chemotactic response to zymosan-activated serum or C5a without affecting the response to FMLP (22). The diminished PMN response to complement-derived stimuli in vitro correlated with an inhibition of PMN migration in a dermal reversed passive Arthus reaction. At least four mechanisms could account for this effect. These include stimulus-specific desensitization, a shift in PMN subpopulations, a direct effect of LPS itself, or an indirect effect of LPS

TABLE 3. Effect of plasma from LPS-injected rabbits on PMN chemotactic responses

Plasma concn, time after LPS treatment	Change in net PMN migration (μm/35 min) after incubation with[a]:		
	FMLP	C5a	LTB
25% plasma			
1 h	−3.2 ± 1.7 (5)	−22.7 ± 1.7 (4)[b]	−18.1 ± 1.7 (5)[b]
24 h	0.2 ± 1.9 (5)	−19.7 ± 3.0 (5)[b]	−0.3 ± 1.4 (4)
10% plasma			
1 h	−5.8 ± 3.8 (13)	−4.3 ± 7.0 (11)	−12.4 ± 2.9 (14)[b]
24 h	−8.0 ± 4.0 (10)[c]	−4.1 ± 4.6 (11)	−7.8 ± 2.7 (10)[d]

[a] Results (mean ± standard error) are expressed as change in net migration relative to the migration of PMN incubated in a comparable concentration of normal plasma. Values in parentheses give the number of paired plasma examined. Statistical comparisons are based on a paired t test. None of the incubation conditions had a significant effect on migration in response to buffer alone. Concentrations for each ligand varied slightly between studies but were, of course, equal for paired variables and always in the optimal-to-suboptimal range.

[b] $P < 0.005$.

[c] $P < 0.05$.

[d] $P < 0.01$.

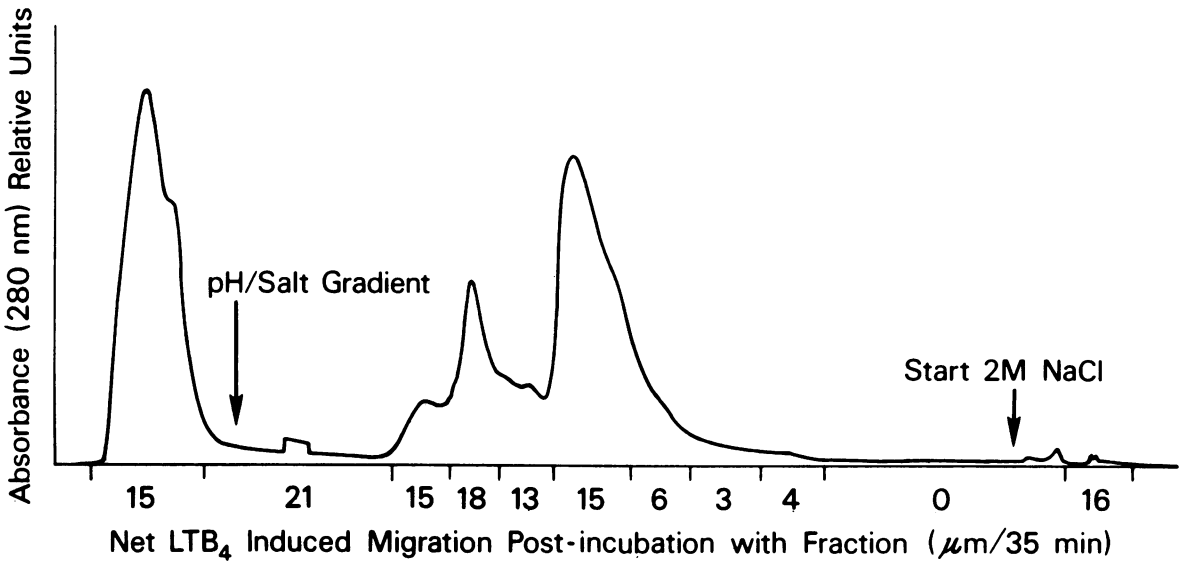

FIG. 2. EDTA plasma (23 ml) was obtained from a rabbit 1 h after LPS injection, dialyzed against a 10 mM phosphate buffer (pH 7.5), and then loaded on a DEAE-cellulose column. The column was eluted first with 10 mM phosphate buffer, then with a pH and salt gradient to pH 6.5 (300 mM), and finally with 2M NaCl. Fractions were monitored for A_{280}, pooled, and dialyzed against phosphate-buffered saline. Normal rabbit PMN were incubated with phosphate-buffered saline or pooled column eluates. PMN incubated in phosphate-buffered saline migrated 19 μm/35 min in response to LTB_4 (5 ng/ml). PMN incubated in column fractions eluting just after the last major peak (albumin) had consistently inhibited PMN responses. Anionic substances eluted in these fractions.

due to the production of a plasma factor or factors. The present data support the last possibility but fail to exclude totally a partial role for other mechanisms.

Stimulus-specific desensitization is a selective unresponsiveness of PMN to a chemotactic factor induced by a prior exposure to this factor (19). It has been described in in vitro and in vivo studies (19, 26). Since endotoxin activates complement through the classical and alternative pathway, C5a should be produced. PAF and LTB_4 synthesis might occur indirectly as a result of C5a-induced leukocyte activation (15). PDGF release should occur as a result of endotoxin-induced platelet secretion (17). Thus, stimulus-specific desensitization is a plausible explanation for our results. However, we have tested the plasma from endotoxin-treated rabbits for its chemotactic activity. We have been unable to show any activity between 5 min and 24 h after the endotoxin injection. Furthermore, we have found no increase in LTB_4 levels in plasma of endotoxin-treated rabbits. (Radioimmunoassay was a courtesy of Edward Goetzl, University of California, San Francisco. Assay sensitivity is 0.01 pm/ml.) These findings and the fact that stimulus-specific desensitization is a transient phenomenon cast doubt on desensitization as an explanation of the detected PMN dysfunction.

Injection of endotoxin induces a shift towards immature forms of PMN. PMN are known to be heterogeneous with respect to chemotactic responsiveness (24). It remains to be shown whether the younger PMN subpopulations have a stimulus-specific defect in their responses. A neutropenia and subsequent neutrophilia can be induced by the intravenous infusion of FMLP. During the neutrophilia, these PMN are not impaired in their response to C5a (J. T. Rosenbaum, unpublished data).

In vitro endotoxin itself is able to inhibit or enhance PMN chemotaxis depending on endotoxin preparation and concentration used (3, 9, 10, 21). In vitro exposure to endotoxin could readily have occurred during the preparation of PMN for our studies. However, exposure to LPS in vitro should have been equivalent for PMN from control and LPS-injected rabbits. Furthermore, selectivity in the altered responses to the chemotactic stimuli comparable to our findings has not been described as an in vitro effect of endotoxin. *Limulus* assay had failed to detect any LPS in the plasma at the time that PMN were obtained for these studies. It still remains possible that an LPS metabolite which does not contain lipid A and thus does not induce gelation in the *Limulus* assay could contribute to our observations.

The transferability of the inhibition of PMN responses to C5a and LTB_4 by plasma supports the role of an endotoxin-induced plasma factor or factors. Responsiveness to FMLP was inhibited only by a 10% concentration of plasma 24 h after treatment with LPS. This inhibition was at a low level of statistical significance, a level of significance frequently noted when a large number of statistical comparisons are performed. The inhibition of responses to LTB_4 but not C5a at a 1:10 dilution of plasma could be reconciled if the inhibitors of C5a and LTB_4 responses are not identical or if additional factors are regulating the function of a possible common inhibitor. An interaction between an inhibitor and a substance regulating that inhibitor might also account for the inhibition of responses to LTB_4 with a 10% but not a 25% concentration of plasma 24 h after treatment with LPS. The possibility of different inhibitors for LTB_4 and C5a is compatible with the data from column chromatography. However, the data obtained from column chromatography must be considered to be preliminary. Further studies will be needed to determine whether normal plasma also contains this inhibitor. If present in normal plasma, LPS-induced changes in responsiveness to LTB_4 could be due to effects on a regulatory substance which controls the inhibitor of LTB_4 responses. Furthermore, in vitro complement activation as could arise from dialysis could contribute to these observa-

tions. For example, Ruddy et al. (23) have described the inhibition of chemotactic responses to C5a and kallikrein by products of activation of the alternative complement pathway.

The discrepancy between the endotoxin-induced effects on PMN migration and secretion is of interest. There are receptors with two different affinities for LTB_4 on the surface of PMN, whereas C5a receptors are all of equal affinity (7). Furthermore, the high-affinity receptors for LTB_4 seem to transduce the chemotactic response while the low-affinity receptors control the secretory response (7). If endotoxin alters PMN responsiveness at a receptor level, down regulation of C5a receptors would alter both migration and secretion while down regulation of the high-affinity LTB_4 receptors would be selective for the chemotactic function. Data from our laboratory support this hypothesis (J. T. Rosenbaum, H. Enkel, D. E. Chenoweth, and D. W. Goldman, Fed. Proc. **44**:994, 1985).

In summary, our results indicate that the responses of PMN to endogenous mediators of inflammation are selectively impaired by intravenous injection of endotoxin, whereas their responses to the synthetic peptide FMLP are unchanged. The transferability of this inhibition by plasma suggests the presence of a soluble inhibitor(s) in the plasma of the endotoxin-treated rabbits. These findings may be related to the altered PMN function that has been associated with gram-negative sepsis.

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