Endotoxin-Associated Protein: a Potent Stimulus for Human Granulocytopoietic Activity Which May Be Accessory Cell Independent

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Proteins coextracted with endotoxin, termed endotoxin-associated protein (EAP), have been shown to exert interleukin 1-like activities. The present studies demonstrate that EAP also exerts potent granulopoietic colony-stimulating activity (CSA) on human peripheral blood and bone marrow progenitor cells, comparable to that seen with various types of conditioned media. The CSA observed with EAP appeared to be heat (100°C, 30 min) and trypsin resistant and partially pronase resistant. Similar resistance was observed with the porin proteins of the outer membrane of gram-negative bacteria, and similar CSA activity was observed with a purified porin preparation of *Neisseria gonorrhoeae*. The CSA of EAP could be demonstrated in human peripheral blood and bone marrow leukocytes rigorously depleted of monocytes, T lymphocytes, and B lymphocytes by treatment with specific monoclonal antibodies and complement.

Endotoxin or lipopolysaccharide (LPS), derived from gram-negative bacilli, has been shown to be a potent stimulus for both human and rodent hematopoietic cells. In vivo and in vitro granulopoietic stimulation by LPS has been believed to involve mechanisms which induce colony-stimulating activity (CSA) production by accessory cells such as monocytes and T cells (3, 12, 31, 32).

A variety of endotoxin (LPS) preparations from numerous bacterial species have been utilized in many studies on the assumption that the biologic activity of all LPS is similar. Various lots of commercial LPS preparations from the same bacterial species and serotype may differ severalfold in biologic activity and exhibit a wide range in degree of contamination by protein and nucleic acids (22). Classically, LPS prepared by the trichloroacetic acid precipitation method described by Boivin et al. contains large amounts of protein (up to 20%) (7, 22), whereas LPS prepared by hot phenol-water extraction may be virtually protein free, although there is some variation with different preparations (35). Studies indicating that proteins, endotoxin-associated proteins (EAPs), contaminating many preparations of LPS and lipid A were potent mitogens for murine and human B lymphocytes (17, 26, 33, 34) prompted investigations which demonstrated that EAP exerted potent interleukin-1 (IL-1)-like activity in the lymphocyte-activating factor and resting T-cell costimulation assays and in inducing production of serum amyloid A (20). The present studies extend these evaluations of the biologic activity of EAP and demonstrate that EAP is a potent granulopoietic stimulus for normal human progenitor cells (CFU of granulocytes and monocytes) and that stimulation of colony formation may occur in the absence of recognizable accessory cells.

MATERIALS AND METHODS

Preparation of EAP. EAP preparations used in these studies were obtained by hot phenol-water extraction (35) of trichloroacetic acid precipitates (7) of *Salmonella minnesota* S218. The EAP was precipitated from the phenol phase with 75% ethanol at -18°C as previously described (20). Assays of endotoxin contamination of EAP demonstrated less than 0.007% LPS by weight, using the actinomycin D mouse lethality assay (28), and less than 0.002% by the *Limulus* amoebocyte lysate assay. This small degree of LPS contamination represents an insufficient quantity of LPS to stimulate colony formation in this system. The EAP preparations were demonstrated to contain several protein bands identifiable by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as outer membrane proteins, as described in the preceding paper and other publications (15, 16, 20).

Soft agar cultures. Whole venous blood or marrow aspirates were collected in preservative-free heparin from healthy volunteers. Whole mononuclear cells were separated from blood or marrow samples on Ficoll-Hypaque gradients. After washing three times in alpha minimal essential medium (MEM) (Flow Laboratories, McLean, Va.) containing 20% fetal calf serum, cells were added to plastic petri dishes to deplete the whole mononuclear cells of monocytes by two sequential 1-h incubations (5). Feeder layers were prepared with concentrations of EAP ranging from 0.1 to 10 µg/ml in 15% fetal calf serum and 0.5% agar in alpha MEM. Overlay layers were prepared with 5×10^5 peripheral blood nonadherent cells (NAC) or 2×10^5 marrow NAC in 1 ml of 15% fetal calf serum and 0.3% agar in alpha MEM. Triplicate cultures were incubated for 14 days. Overlay layers were separated from the feeder layers, stained with 0.1% Luxol Fast Blue, counterstained with hematoxylin, and scored at $\times 100$ magnification. Colonies were defined as discrete collections of 40 or more cells (5).

Preparation of conditioned media. Leukocyte-conditioned medium (LCM) was prepared by using a modification of the

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TABLE 1. Comparative CSA of EAP

Feeder layer		Total colonies/ 5×10^5 NAC ^a (% eosinophils)		
		Peripheral blood NAC ^b		Bone marrow NAC
Medium	Concn per ml of layer	Expt 1	Expt 2	
Control (MEM)	0.1 ml	0 (30)	5 $\pm$ 6 (30)	ND
LCM	0.1 ml	6.5 $\pm$ 1 (30)	32.3 $\pm$ 8 (50)	ND
EAP-LCM ^c	0.1 ml (of 5.0 μ g/ml)	21.0 $\pm$ 1.5 (30)	41.3 $\pm$ 5.5 (57)	ND
Control (MEM)	0.1 ml	1 $\pm$ 0.3 (0)		0
EAP-LCM ^c	0.1 ml (of 5.0 μ g/ml)	38 $\pm$ 2 (71 $\pm$ 12)		170 $\pm$ 6 (0)
EAP alone	0.5 μ g	43 $\pm$ 5 (79 $\pm$ 3)		168 $\pm$ 8 (0)

^a Mean of triplicate values $\pm$ standard deviation. ND, Not done.

^b Experiments were carried out with blood from different donors.

^c EAP added at 5.0 μ g/ml to LCM; final concentration in feeder layer, 0.5 μ g/ml.

method described by Iscove et al. (19). Whole mononuclear cells, collected as described above, were incubated for 7 days in 5% CO₂ at 37°C at a concentration of 10⁶ cells per ml in alpha MEM containing 20% fetal calf serum and 0.5 mM β -mercaptoethanol. After 7 days of incubation, the culture was centrifuged at 2,000 $\times$ g for 15 min, and the supernatant was collected and concentrated 12-fold on an Amicon ultrafiltration cell (Diaflo PM 10 membrane). The concentrate was passed through a 0.22- μ m-pore-size membrane filter (Millipore Corp., Bedford, Mass.) and stored at 0°C for further use. Titration revealed maximal CSA with 0.1 ml of LCM per ml of feeder layer. Phytohemagglutinin LCM (PHA-LCM) was prepared in a similar fashion except that 1% PHA was added to the whole mononuclear cell preparation. EAP-LCM was prepared by adding various concentrations of EAP to the medium before addition of and incubation with the mononuclear cell cultures. Giant-cell conditioned medium (GCCM) was purchased from a commercial source (GIBCO Laboratories, Grand Island, N.Y.).

Heat, trypsin, and pronase treatment of EAP. Solutions of EAP (0.1 mg/ml) were heated in water baths at temperatures of 56, 80, and 100°C for 30 min to evaluate the effects of heat on granulopoietic stimulating activity.

EAP was also treated with trypsin and pronase to determine the effects of these proteolytic enzymes on biologic activity. Samples of EAP (1 mg/ml) were treated with 35 μ g of trypsin (2 $\times$ recrystallized; Sigma Chemical Co., St. Louis, Mo.) or pronase (grade B; Calbiochem, La Jolla, Calif.) per ml at 37°C for 24 h at the pH optimum for their proteolytic activity. Digestive activity of trypsin was blocked by soybean trypsin inhibitor (Aldrich Chemical Co., Milwaukee, Wis.), and pronase activity was blocked by heating at 90°C for 45 min. All components of the reaction mixtures were subsequently tested for CSA.

Gonococcal porin (protein I) preparation. A limited amount of a preparation of porin (protein I, an outer membrane protein from *Neisseria gonorrhoeae*, purified by the method of Blake and Gotschlich [6]), was kindly provided by Peter Rice. This was prepared by treatment of *N. gonorrhoeae* in 2% hexadecyltrimethylammonium bromide (Sigma), which precipitates protein I at low ionic strength but solubilizes most other contaminating proteins. The precipitate, suspended in water, was treated with absolute ethanol to a concentration of 20% to precipitate nucleic acids. Protein I was precipitated by further addition of ethanol to 80%, washed, and suspended (2%, wt/vol) in 50 mM Tris hydrochloride (pH 8.0) with 10 mM EDTA, 0.5 M NaCl, and 5% *N*-tetradecyl-*N,N*-dimethyl-3-ammonia-1-propanesulfonate (Zwittergent 3-14; Calbiochem). This precipitate was se-

quentially chromatographed on DEAE-Sepharose 6B-CL (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) and Sephacryl S-300 (Pharmacia), and fractions containing the porin were pooled in 100 mM Tris hydrochloride–20 mM NaCl–10 mM EDTA–0.05% Zwittergent at pH 8.0. Repeated dialysis of the preparation failed to completely remove the Zwittergent 3-14, leaving a residual content of 0.00016%.

Accessory cell depletion. Ficoll-Hypaque-separated whole mononuclear cells were depleted of monocytes by two sequential 1-h incubations in plastic petri dishes. T cells were removed by rosetting twice with 2-aminoethylisothiourea bromide hydrobromide-treated sheep erythrocytes (21), and the nonrosetting fraction was collected. After these procedures, the preparation contained less than 2% nonspecific esterase-positive cells and fewer than 1% OKT3-positive cells. To further deplete the population of accessory cells, monoclonal antibodies (MAbs) and complement were employed. The cells were treated with two anti-T-cell antibodies (10.2 [23] and 9.6 [18]), two antimonocyte antibodies (5F1 and T5A7 [20]), two anti-B-cell antibodies (2H7 and 1F5 [10]), and prescreened rabbit serum as a source of complement. After this treatment, the cell preparation was studied by fluorescence-activated cell sorter analysis to determine whether any T cells, B cells, or monocytes were detectable with the antibodies noted above. The monocyte-, T-cell-, and B-cell-depleted preparation was tested for colony growth using EAP alone and a potent PHA-LCM.

RESULTS

CSA of EAP. Initial studies of the CSA of EAP evaluated the ability of the protein to enhance the activity of various types of conditioned media (LCM [19] and PHA-LCM [32]), whole mononuclear cell layers (29), and GCCM (13), previously demonstrated to have potent CSA. Table 1 illustrates two representative experiments utilizing peripheral blood from two different volunteers to compare the CSA of 0.1 ml of LCM alone and that of 0.1 ml of LCM to which EAP had been added to a final concentration of 5.0 μ g/ml before incubation with the mononuclear cell cultures. Little, if any, colony formation was observed with control cultures of peripheral blood. This demonstration that EAP enhanced the CSA of conditioned media prompted examination of the CSA of EAP alone. The CSA of 0.5 μ g of EAP alone was comparable to that of EAP-LCM (with identical final EAP content) on peripheral blood and bone marrow leukocytes (Table 1).

Whole agar plates were stained with Luxol Fast Blue and hematoxylin to determine the types of colonies stimulated by

FIG. 1. Dose-response curve of colony formation in response to various concentrations of EAP.

EAP (5). Colonies derived from peripheral blood NAC contained a substantially larger number of eosinophil colonies, $\geq 33\%$, whereas those derived from bone marrow cells were predominantly monocyte-polymorphonuclear leukocyte colonies. These findings are similar to earlier observations that culture of peripheral blood NAC with a source of CSA results in a much higher proportion of eosinophil colonies than bone marrow NAC. However, EAP consistently produced a higher proportion of eosinophil colonies than did other granulopoietic stimulants. Since individual normal donors were utilized as sources of peripheral blood or bone marrow NAC, the degree of stimulation varied with the leukocytes from these individuals, but CSA which significantly exceeded that of control media and was comparable to or greater than that of the various conditioned media tested was uniformly observed with NAC from all normal donors after stimulation with EAP. Similar results were obtained with three different lots of EAP, supporting the reproducibility of these results.

A dose-response curve of the CSA of EAP is illustrated in Fig. 1. Maximal stimulation occurred with EAP concentrations of $0.5 \mu\text{g/ml}$, with a lesser response being noted at concentrations of $\geq 1.0 \mu\text{g/ml}$. All subsequent experiments were performed with $0.5 \mu\text{g}$ of EAP per ml, a concentration at which colony formation with EAP alone was equivalent to or greater than that of other potent sources of CSA including PHA-LCM (5), LCM (19), whole mononuclear cell feeder layers (29), and GCCM (13). Several experiments were carried out using purified *S. minnesota* LPS to ensure that the minimal contamination of EAP with LPS was not responsible for the CSA observed. No CSA could be detected with concentrations of $< 5 \mu\text{g}$ of *S. minnesota* LPS. Similarly, the addition of 10 U of polymyxin B failed to inhibit the CSA of $0.5 \mu\text{g}$ of EAP.

Stability of EAP to trypsin, pronase, and heat. The stability of EAP to treatment with the proteolytic enzymes trypsin and pronase and heat denaturation was evaluated (Table 2). Treatment with trypsin failed to diminish the CSA of 0.1 ml ($5 \mu\text{g/ml}$) of EAP as compared with the activity of untreated EAP. Pronase treatment was associated with a reduction in CSA activity in comparison with that observed with untreated EAP. The granulopoietic activity of EAP was relatively resistant to heating at 56, 80, or 100°C for 30 min. Although there was slight but progressive decrease in CSA with heating at increasing temperatures, EAP heated to 100°C for 30 min was still capable of stimulating considerable proliferation of granulopoietic colonies, in comparison with the number of colonies observed with alpha MEM controls, and was still capable of inducing approximately 70% as many colonies as untreated EAP.

CSA of porin preparation. The resistance to trypsin, partial resistance to pronase, and relative resistance to

TABLE 2. Effects of heat and enzyme treatment on EAP^a

Feeder layer (treatment)	No. of colonies/ 5×10^5 peripheral blood NAC
Control (0.1 ml of alpha MEM)	4 ± 1.0
EAP ($5.0 \mu\text{g/ml}$)	58 ± 12
EAP + pronase ($5.0 \mu\text{g/ml}$)	29 ± 15
EAP + trypsin ($5.0 \mu\text{g/ml}$)	61 ± 5
Control (0.1 ml of alpha MEM)	5 ± 0.2
EAP ($5.0 \mu\text{g/ml}$)	74 ± 15
EAP ($5.0 \mu\text{g/ml}$, 56°C , 30 min)	61 ± 11
EAP ($5.0 \mu\text{g/ml}$, 80°C , 30 min)	57 ± 4
EAP ($5.0 \mu\text{g/ml}$, 100°C , 30 min)	53 ± 14

^a The results of each experiment represent the mean $\pm$ standard deviation of triplicate plates.

heating to 100°C for 30 min is unique to the porins of the outer membrane proteins of gram-negative bacteria. Since there is considerable chemical and immunologic similarity between the porins of different species of gram-negative bacteria, the availability of a purified porin preparation (generously supplied by Peter Rice) prompted evaluation of its CSA (Fig. 2). Initial studies were complicated by the inability to completely remove the Zwittergent and the extreme toxicity of this compound for eucaryotic cells: 0.0016% Zwittergent markedly inhibited the CSA of both LCM and EAP. Serial dilution of the porin preparation demonstrated significant CSA of the porin preparation at a concentration of 10^{-7} and a Zwittergent concentration of 10^{-10} . Smaller concentrations of the porin preparation failed to demonstrate CSA presumably because they were below the threshold concentration for CSA.

Accessory cell depletion. In five experiments with peripheral blood (Table 3) and four experiments with marrow (Table 4) from different donors, mononuclear cells, depleted

FIG. 2. CSA of gonococcal porin containing small amounts of Zwittergent. Zwittergent inhibited the porin CSA until the preparation was diluted to a concentration of 10^{-7} of the porin and 10^{-10} of the Zwittergent. No CSA was observed with lower concentrations of the porin. Zwittergent also inhibited the CSA of EAP and LCM at a concentration of 10^{-5} . PBNAC, Peripheral blood NAC.

TABLE 3. Effect of EAP on peripheral blood granulocytic colony growth^a

Cells	No. of colonies/ 5 × 10 ⁵ cells		% Eosinophils	
	Control medium	+EAP	Control medium	+EAP
Peripheral blood	0	111 ± 9		65 ± 3
NAC	0	11 ± 1		44 ± 14
	0	19 ± 3		33 ± 11
	1 ± 1	43 ± 8	0	79 ± 11
	3 ± 1	52 ± 5	0	51 ± 2
Mean	1 ± 1	41 ± 39	0	54 ± 18
T-cell-depleted	0	170 ± 30		61 ± 15
peripheral	0	10 ± 1		50 ± 10
blood NAC	0	27 ± 3		43 ± 7
	0	102 ± 5		71 ± 17
	0	87 ± 6		52 ± 5
Mean	0	79 ± 64		55 ± 11

^a Mean ± standard deviation of triplicate plates counted after staining with Luxol Fast Blue and hematoxylin. Each experiment used cells from a different donor.

of adherent cells, were stimulated by EAP to produce an average of 41 ± 39 granulocytic colonies per 5 × 10⁵ peripheral blood NAC (Table 3) and 109 ± 31 colonies per 2 × 10⁵ marrow NAC (Table 4). Peripheral blood and marrow NAC cultured in the absence of an exogenous source of CSA produced three or fewer colonies per 5 × 10⁵ NAC. Cultures of peripheral blood stimulated with EAP contained 54 ± 18% eosinophil colonies (Table 3); the remainder were neutrophil or neutrophil-monocyte colonies. In contrast, marrow cultures contained only 6 ± 6% eosinophil colonies and 94 ± 6% neutrophil-monocyte colonies (Table 4). The proportion of eosinophil colonies seen after stimulation of peripheral blood with EAP was somewhat greater than the ratio of eosinophil to neutrophil or neutrophil-monocyte colonies seen with other sources of CSA (5).

T cells and monocytes are both capable of releasing CSA when appropriately stimulated (9, 11). To further test whether EAP was inducing CSA production by these cells and thus indirectly stimulating colony formation, mononuclear cell preparations were rigorously depleted of monocytes and T cells by incubation in plastic petri dishes and aminoethylisothiuronium bromide hydrobromide-treated sheep erythrocyte rosetting. The T-cell-depleted NAC contained less than 2% nonspecific esterase-positive cells.

TABLE 4. Effect of EAP on bone marrow granulocytic colony growth^a

Cells	No. of colonies/ 2 × 10 ⁵ cells		% Eosinophils	
	Control medium	+EAP	Control medium	+EAP
Bone marrow NAC	0	75 ± 7	0	10 ± 4
	0	118 ± 17	0	1 ± 1
	0	134 ± 11	0	5 ± 4
Mean	0	109 ± 31	0	6 ± 6
T-cell-depleted bone marrow NAC	0	168 ± 14	0	1 ± 1

^a Mean ± standard deviation of triplicate plates counted after staining with Luxol Fast Blue and hematoxylin. Each experiment used cells from a different donor.

Fewer than 1% of NAC reacted with OKT3 in an indirect microscopic immunofluorescence assay. In five experiments (Table 3), no loss of EAP growth stimulation was noted after T-cell depletion. To further exclude EAP-mediated accessory-cell CSA production, a cocktail of anti-T-cell (10.2 [23] and 9.6 [18], antimonocyte (5F1 and T5A7 [1]), and anti-B-cell (2H7 and 1F5 [10]) MAbs, with prescreened rabbit serum as a source of complement, was used to treat the T-cell-depleted NAC. After this treatment, fluorescence-activated cell sorter analysis demonstrated the absence of T cells detectable with MAbs 9.6 and 10.2, B cells detectable with 2H7 and 1F5 MAbs, or monocytes detectable with the 5F1 MAb. Nevertheless, when this cell population was tested for granulocytic colony growth, EAP stimulated numbers of colonies (45 ± 11) equivalent to those stimulated by a potent PHA-LCM (44 ± 8). (Controls produced 6 ± 2 colonies.) This evidence strongly suggests that EAP may act independently of accessory cells by directly stimulating progenitor cells.

DISCUSSION

Development of cloning techniques and in vitro culture methods has greatly enhanced the delineation of mechanisms involved in hematopoietic differentiation (8, 30). These techniques have allowed the demonstration that incubation of buoyant peripheral blood or marrow mononuclear cells for 14 days in semisolid support medium with a colony-stimulating factor (CSF) as a source of CSA resulted in growth of colony forming units in culture. The continued presence of CSA is necessary for division of cells committed to granulocyte macrophage production. Several CSFs, which belong to a family of glycoproteins, have been identified as being produced by a variety of cell types (T lymphocytes, monocytes, fibroblasts, and vascular endothelial cells) and to exert differential stimulatory activity on various progenitor cells. Cellular sources, such as LCM, PHA-LCM, GCCM, and cell feeder layers, were usually used as sources of CSF for studies of granulopoiesis before purified CSFs were produced by cloning and genetic engineering. These various types of conditioned media differ in their ability to stimulate proliferation of different cell lines; e.g., PHA-LCM actively stimulates eosinophil proliferation, whereas GCCM favors monocyte growth, ostensibly due to the presence or relative content of various CSFs. EAP represents another material which has potent eosinophil CSA.

Bacterial products, of which LPS has been most extensively studied, have been implicated as having profound effects in vitro and in vivo on granulopoiesis (reviewed in references 24 and 25). Most studies have suggested that LPS or its lipid A component does not exert a direct effect on granulocytic progenitor cells but that the granulopoietic effects of LPS result from stimulation of monocytes or other cells to release CSF or other cytokines which, in turn, induce CSA (2, 12, 14, 31, 32). Recent studies indicate that LPS exerts its granulopoietic activity through its potent stimulatory effect on monocyte IL-1 production and IL-1-induced release of granulocyte-macrophage CSF from other cells (6). This demonstration of the striking granulopoietic stimulating activity of EAP provides further evidence of IL-1-like activity of this component of the cell wall of gram-negative bacilli. However, the present studies differ from observations of the granulopoietic effects of IL-1 in suggesting that EAP exerts a direct effect on the maturation of progenitor cells. It could be argued that, despite rigorous

depletion of monocytes and T and B lymphocytes, some cells remained that were capable of producing CSF. The inability to detect residual cells with markers for monocytes and T and B lymphocytes by fluorescence-activated cell sorter analysis after depletion excludes this argument as well as is possible. In addition, the failure of rigorous depletion of these cell types even to reduce the stimulatory effects of EAP indicates that if residual cells were responsible for the observed CSA, the cells responsible for this activity apparently do not bear antigenic markers recognized by the MABs utilized. It is anticipated that future studies will definitely establish whether EAP has direct CSA or acts by stimulating other peripheral blood or marrow NAC to produce CSA. Irrespective of whether EAP directly stimulates precursor cells or acts through CSF induction, it does represent a unique granulopoietic stimulus derived from procaryotic cells which is extremely active at low concentrations.

EAP growth-stimulating activity was resistant to heat denaturation (100°C for 30 min) and resistant to treatment with trypsin and pronase. These properties are similar to those reported for the bacterial cell wall proteins known as porins, which produce water-filled diffusion channels through membranes, have been reported to be mitogenic for mammalian lymphocytes (4, 27), and comprise up to 45% of EAP (15). The CSA observed with a porin preparation from *Neisseria gonorrhoeae* suggested that it may be the primary granulopoietic stimulating factor present in EAP, but the cytotoxicity of the detergent required for porin purification hampered definitive proof of this activity. Attempts at isolation and identification of the active component of EAP by techniques which do not utilize cytotoxic detergents are in progress. However, irrespective of identification of the specific active component, these studies do indicate that EAP, a non-eucaryotic cell product, stimulates the production of granulocytes, a cell population whose primary function is destruction of bacterial pathogens.

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