Chemotactic factor-enriched butanol extracts from *Escherichia coli* culture filtrates were fractionated and purified by high pressure liquid chromatography. The yield from individual fractions of biological activity (lysosomal enzyme secretion) and antigenic activity (competition with \[^3H\]Met-Leu-Phe for binding to rabbit anti-Met-Leu-Phe) revealed an average 50% recovery of original material. Five peaks of biological activity were separated as demonstrated by enzyme-releasing activity. Three of these peaks coincided exactly with peaks of antigenic activity, suggesting that at least 3 and as many as 5 distinct formyl-methionyl peptides had been separated. The majority of recovered activity appeared in peak 3 and represented 70% of the total biological and antigenic activities recovered. The five peak fractions were subsequently analyzed by dipeptidyl carboxypeptidase gas chromatography-mass spectrometry (DCP/GC-MS) to determine amino acid sequences. After digestion, the formyl-Met peptide was demonstrated in only one of the five peak fractions (peak 3). Furthermore, both the GC retention times and mass spectra indicated that peak 3 contained for- myl-methionyl-leucyl-phenylalanine. The DCP/GC and MS data were confirmed with tests made on authentic fMet-Leu-Phe. Butanol extracts from *E. coli* filtrates to which were added synthetic fMet-Leu-Phe resulted in increased biological and antigenic activity in the precise high pressure liquid chromatography fractions of peak 3 where the fMet-Leu-Phe produced by *E. coli* was found. Finally, the analysis of recovered biological and antigenic activities indicated that the formyl peptides were found in nanomolar concentrations in culture filtrates. These results demonstrate that the NH₂-terminal formyl peptides produced by *E. coli*, of which formyl-methionyl-leucyl-phenylalanine appears to be the major component, are the peptide mediators responsible for leukocyte chemotactic activity in the bacterial culture extracts.

It has long been known that bacteria, such as *Escherichia coli*, elaborate a number of extremely potent chemotactic substances in cell culture medium (1, 2). The detailed structural analysis of these substances, however, has been hampered by their presence in very low concentrations. Partial characterization of culture filtrates from *E. coli* has established that some of the active components are small heterogeneous peptides with blocked amino groups (3). This work, as well as the recognition that a number of bacteria elaborate similar chemotactic substances, suggested that a specific class of compounds characteristic of these cells could be involved. Such prokaryotic cell products could consist of formylmethionyl peptides derived from the NH₂-terminal regions of newly synthesized proteins (4). Since leukocytes accumulate at foci of bacterial infections, they may, as eukaryotic cells, be responding in part to such a typically prokaryotic cell material. This hypothesis has been supported by the finding that simple formylmethionyl peptides are potent chemoattractants for leukocytes (4). Other indirect evidence that these attractants are formylmethionyl derivatives has come from both receptor-binding (5) and immunochemochemical (6, 7) data. The chemical properties of the bacterial factors and the synthetic peptides are also similar (3).

While it has been attractive to propose that eukaryotic cells recognize prokaryotic cell products derived from NH₂-terminal regions of nascent proteins, there has been no direct evidence for this. This paper will establish for the first time that formyl peptides are the natural chemotactic products of bacteria. Furthermore, we will show that the tripeptide formyl-Met-Leu-Phe, the synthetic analogue of which was synthesized almost a decade ago (8) and whose radiolabeled derivative, fMet-Leu-[\(^3H\)]Phe, has been used extensively to characterize the receptor for the formyl peptides on phagocytic cells (5, 9–15), is the major peptide neutrophil chemotactic factor produced by *E. coli*.

**EXPERIMENTAL PROCEDURES**

**Materials**

The synthetic peptides fMet-Leu-Glu, fMet-Gly-Phe, fAla-Leu-Phe, and Met-Leu-Phe were generous gifts from Dr. Richard J. Freez, Department of Pharmacology, Medical College of Virginia. Formyl-methionyl-leucyl-phenylalanine, p-nitrophenyl-N-acetyl-β-D-glucosaminide, oyster glycogen, and rabbit lung acetone powder were purchased from Sigma. Cytochalasin B was purchased from Aldrich. The radiolabeled peptide, fMet-Leu-[\(^3H\)]Phe (47.6 Ci/mmol) was obtained from New England Nuclear.

**Methods**

Preparation of Partially Purified Chemotactic Factors from *E. coli* Culture Filtrates—The complete protocol for the isolation and partial

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characterization of this material has been previously reported (3), and it represents the material used by the University of Connecticut group (procedure II). In brief, filtrate (250 liters) from a 24-h culture of *E. coli* WRAIR 1485 (obtained from Walter Reed Army Institute of Research) in minimal media was extracted twice with water-saturated butanol in a liquid extractor. The 49 liters of the first extract and 39 liters of the second were combined and concentrated to 1 liter, which was stored at −70 °C. The further characterization of this crude butanol extract (ion exchange, gel filtration, gel electrophoresis, and amino acid analysis) has been previously reported (3).

**High Pressure Liquid Chromatography of Partially Purified *E. coli* Culture Filtrates—**In several experiments, 1 to 3 ml of partially purified material was fractionated into bacterial chemotactic factor (from above) were dried in a Speed Vac (Savant Instruments) rotary evaporator and resuspended in the HPLC buffer. The liquid chromatography system consisted of a Varian LC-5020 chromatograph and Varichrom variable wavelength detector. The sample was run through a reverse phase C18 10-μm (MCH10), or 5-μm (MCH5) particle-size column (Varian Instruments). Chromatography was run under the following conditions: flow rate, 1.0 ml/min; temperature, 30 °C; gradient elution from 20 to 45% CH3CN in H2O containing 0.1% CF3COOH throughout, over 60 min. Fractions were collected every minute (unless otherwise specified) and thoroughly dried in a Speed Vac rotary evaporator, resuspended to 0.5 ml in H2O, and assayed for biologic activity. Spectrophotometric detection was set at 210 nm. Under these conditions, column pressure never exceeded 250 atm.

**Analysis of Recovery of Biological Activity from Individual HPLC Fractions—**For each biological assay, the per cent of recovered material in each peak was then determined. The per cent of inhibition data from the individual fractions, and these values from spectrometry; SBF, stock bacterial chemotactic factor; EDsa, equivalents from the per cent of specific enzyme release data for the fractions of recovered bacterial chemotactic factor. These values from each HPLC fraction were then summed and multiplied by the fitted curve from (O,O), and the minimum aliquot of this latter ACE preparation necessary for efficient digestion of fMLF was used. These experiments had the added benefit of providing authentic mass spectra and gas chromatographic elution temperatures for formyl-Met and Leu-Phe for as much as possible, and the minimum aliquot of each of these fractions to competition with Met-Leu-Phe for binding to rabbit anti-Met-Leu-Phe, 50-pl aliquots of each HPLC fraction, 50 μl containing 88 fmol of Met-Leu-[3H]Phe, and 50 μl of an appropriate dilution of rabbit anti-Met-Leu-Phe were incubated at 24 °C for 30 min and then analyzed for competitive binding as previously described (6). To quantify recovery of SBF using the radiomimimunassay data, injection of tMet-Leu-[3H]Phe was plotted versus micromolars of SBF in a given dilution. Linear regression analysis of the data yielded a standard curve (r = 0.989). Using this best fit line, the per cent of inhibition was converted to micromolars of SBF for individual fractions. These data were corrected for deviation of the best fit line from (0,0), summed, and converted to per cent of recovery of original material. The per cent of recovered material in each peak was then calculated.

where fMLF was not assayed, the mean fMLF data from three similar experiments were used.

**Radioimmunoassay Analysis of Recovery of Antigenic Activity from Individual HPLC Fractions—**To analyze by radioimmunoassay the ability of the individual HPLC fractions to compete with fMet-Leu-[3H]Phe for binding to rabbit anti-fMet-Leu-Phe, 50-μl aliquots of each HPLC fraction, 50 μl containing 88 fmol of Met-Leu-[3H]Phe, and 50 μl of an appropriate dilution of rabbit anti-Met-Leu-Phe were incubated at 24 °C for 30 min and then analyzed for competitive binding as previously described (6). To quantify recovery of SBF using the radiomimimunassay data, injection of tMet-Leu-[3H]Phe was plotted versus micromolars of SBF in a given dilution. Linear regression analysis of the data yielded a standard curve (r = 0.989). Using this best fit line, the per cent of inhibition was converted to micromolars of SBF for individual fractions. These data were corrected for deviation of the best fit line from (0,0), summed, and converted to per cent of recovery of original material. The per cent of recovered material in each peak was then calculated.

The fMLF mole equivalents were calculated by fitting per cent of inhibition of [3H]fMLF binding to rabbit anti-fMLF versus fMLF concentration (molarity) data to a curve generated by the modified exponential curve-fitting program in a Sharp computer with a modified exponential curve-fitting program (16). Lyso-
compositions. As shown in Fig. 1, we were able to establish a chromatography system which resulted in marked differences in retention time for several formylated tripeptides. Several additional formylated tri- and tetrapeptides (not shown in Fig. 1) were also analyzed, and their retention times showed essentially less than ±5% variation when comparing individual runs of peptides applied to the column singly or when applied to the column as a mixture. In addition, Met-Leu-Phe could easily be separated from its formylated derivative. Furthermore, under the assay conditions we describe, the HPLC-fractionated peptides remain biologically active and maintain their antigenicity (data not shown). Also evident in Fig. 1 is the fact that as the peptides become more hydrophobic in nature, their retention times are increased considerably. It is well established that both the biological activity of these peptides and their affinity for the formyl peptide receptor increase as the hydrophobic character of the constituent amino acids increases (8, 19, 20). Thus, this chromatography system has several favorable features to allow for separation and purification of bacterial chemotactic factor.

Fractionation of Partially Purified E. coli Culture Filtrate by High Pressure Liquid Chromatography

A. Analysis of Ultraviolet Absorption Profile at 210 nm—To avoid possible trace contamination by \( \text{fMet-Leu-Phe} \) retained on columns during previous runs (Fig. 1), the bacterial chemotactic factor was analyzed using only columns that had not been exposed previously to formylated peptides. As can be seen in Fig. 2, the partially purified bacterial chemotactic factor sample demonstrated numerous peaks throughout the entire 20-45% CH\(_2\)CN elution gradient. Most of the material that eluted early gave the strongest 210-nm absorbance. However, several small peaks were seen at CH\(_2\)CN concentrations >25%, which would correspond to the more hydrophobic and biologically active formyl peptides (see below and Fig. 1).

B. Separation and Recovery of Biological Activity—Analysis of activity in individual HPLC fractions (at a 1:100 final dilution) for their ability to induce lysosomal enzyme release from rabbit neutrophils revealed an average per cent of recovery of antigenic activity seen in Fig. 3A. The largest antigenic peak (Fraction 15) was recovered at a 1:10,000 dilution (data not shown). Detection at 210 nm failed to demonstrate a peak corresponding to the activity of peak 3, suggesting that this peak contained <1 nmol of Met-Leu-Phe (as compared to calibration using pure standards (Fig. 1)). Therefore, for DCP/GC-MS structural analysis, only the central peak fractions were used. Authentic fMet-Leu-Phe, when subsequently applied to the column as a mixture, resolved the starting material into 5 major peaks of activity (Fig. 3A) with retention times of 27, 33, 45, 55, and 69 min, respectively (Fig. 2). The majority of activity appeared in peak 3 (Fractions 21-26), and this was the only peak in which activity remained at a 1:10,000 dilution (data not shown). When assayed at a 1:100 and 1:1000 dilution, peak 3 contained 55 and 69% of the total recovered activity, respectively, whereas peak 4 (Fractions 27-31) contained 32 and 17%, respectively. Peak 1 (Fractions 13-15), peak 2 (Fractions 16-18), and peak 5 (Fractions 33-37) contained activity only at a 1:100 dilution, with each peak representing 5-7% of the total recovered activity. The broad nature of these peaks is due to the extremely high specific activity of this material (5, 8). Control experiments in which the activity profile of Met-Leu-Phe had been assessed in individual fractions after being applied to the column gave a similarly broad peak (data not shown). Detection at 210 nm failed to demonstrate a peak corresponding to the activity of peak 3, suggesting that this peak contained <1 nmol of fMet-Leu-Phe (as compared to calibration using pure standards (Fig. 1)).

C. Separation and Recovery of Antigenic Activity—We have previously reported that this same partially purified butanol extract from E. coli can compete with fMet-Leu-Phe for binding to rabbit anti-fMet-Leu-Phe (6). In addition, anti-fMet-Leu-Phe bound to protein A-Sepharose can remove the biologically active components of this crude extract (6). We, therefore, used our radioimmunoassay to monitor the separation and to quantitate the antigenic recovery of formylmethionyl peptides in the individual HPLC fractions.

Analysis of antigenic activity in the same individual HPLC fractions (above) gave the pattern of reactivity shown in Fig. 3B with an average per cent of recovery of original applied material of 69.2 ± 28.1% (S.E.), with a range 38.4-100% (n = 3). This is in good agreement with the recovery of enzyme-releasing activity (Fig. 3A). Several distinct peaks of activity are demonstrated which overlap with the peaks of biological activity seen in Fig. 3A. The largest antigenic peak (Fraction 29), which contains 66.2% of the recovered antigenic activity,
Purification of Bacterial Chemotactic Factor

Identification of Formyl-Methionyl-Leucyl-Phenylalanine by Dipeptidyl Carboxypeptidase/Gas Chromatography-Mass Spectrometry

The fraction with the most activity within each of the 5 bioactive HPLC peaks was chosen to be further tested for the presence of fMLF using DCP/GC-MS analysis. Only fraction 23 from bioactivity peak 3 contained fMLF. The other 4 fractions, therefore, served as good background controls. The GC-MS data obtained from fraction 23 are presented in Figs. 5, 6, and 7, where they are compared to authentic fMLF. Fig. 4 shows the total ion chromatograms for these two samples, which are equivalent to gas chromatograms. The identity of the labeled peaks in the chromatograms was determined from their mass spectra, which are described when Fig. 6 is considered. The extraneous peaks present in the total ion chromatogram of DCP-digested and TMS synthetic fMLF are contaminants carried over from the synthetic preparation, as expressed as fMLF mole equivalents (see under "Experimental Procedures") is shown in Table I. Good agreement in the overall recovered activity units by both assays is evident with a less than 15% difference between the two methods of measuring recovery. For peaks 1, 3, and 5, good overall agreement is seen, with peaks 3 and 5 showing less than 15% difference and peak 1 showing a 3-fold difference in activity. The major differences, however, are seen in peaks 2 and 4 where a respective 45- and 8-fold greater enzyme-releasing activity versus antigenic activity is seen (see "Discussion"). Based on these fMLF mole equivalents and correcting for an overall 50% recovery of original activity, the estimated fMLF concentration of the partially purified filtrate (starting material) is approximately $2.76 \times 10^{-7}$ M. This value is in good agreement with the ED$_{50}$ of this starting material which is 1:2500-15000. Furthermore, since this starting material was the extract of 250 liters of culture filtrate concentrated to 1 liter (see under "Experimental Procedures"), the estimated concentration of the formyl peptide in the original culture filtrate is, therefore, approximately $1.1 \times 10^{-9}$ M. As we will show (below), however, only approximately 60% of this starting material (peak 3) appears to be naturally occurring fMet-Leu-Phe.

Table I

| Peak | Fraction numbers | Enzyme release assay* | Calculated recovery of fMLF mole equivalents$^a$ | Radioimmunoassay$^b$ | Activity ratio$^c$ (radioimmunoassay/enzyme release) |
|------|-----------------|-----------------------|-----------------------------------------------|---------------------|---------------------------------------------------|
| 1    | 13-15           | 7.51 $\times 10^{-12}$ | 2.39 $\times 10^{-11}$                        | 3.12                |
| 2    | 16-18           | 1.98 $\times 10^{-7}$  | 2.82 $\times 10^{-7}$                        | 0.022               |
| 3    | 21-26           | 1.18 $\times 10^{-12}$ | 1.47 $\times 10^{-12}$                       | 1.25                |
| 4    | 27-31           | 7.58 $\times 10^{-12}$ | 9.7 $\times 10^{-12}$                        | 0.128               |
| 5    | 33-37           | 1.05 $\times 10^{-12}$ | 1.16 $\times 10^{-12}$                       | 1.11                |
| Total|                 | 2.24 $\times 10^{-12}$ | 1.92 $\times 10^{-12}$                       | 0.86                |

$^a$Analysis of lysosomal enzyme-releasing activity in individual HPLC fractions was determined at a 3:100 final dilution.

$^b$For quantification of recovery of biological and antigenic activity and for conversion of quantified recovery into fMLF mole equivalents see appropriate section under "Experimental Procedures."

$^c$Activity ratio is the calculated ratio of recovered fMLF mole equivalents determined by radioimmunoassay divided by fMLF mole equivalents obtained by the enzyme release assay.
formyl-Met, and the ion mass of 158 corresponds to the ion used to search for TMS Leu-Phe. The ion mass of 247 results from loss of CH₃SCH=CH₂ from the molecular ion of TMS fMLF, and thus allow comparison to the elution temperatures for formyl-Met and Leu-Phe, from DCP-digested chromatogram also indicates, from the presence of Phe, that even under the modified digestion conditions, Leu-Phe is partially hydrolyzed by residual dipeptidase present in the DCP preparation used. Leu elutes at approximately 90 °C and thus appears in the solvent front of this chromatogram. Fig. 4 also shows that the total ion chromatogram obtained from DCP-treated and trimethylsilylated HPLC fraction 23 exhibited a number of extraneous peaks. These peaks were also shown in the total ion chromatograms obtained following similar treatment of the other four HPLC fractions. The mass spectra of these peaks generally indicated that they were various siloxanes, presumably from HPLC column bleed, and fatty acids, possibly acquired during sample handling. No further attempts were made to characterize the extraneous peaks appearing in the chromatograms in Fig. 4. The total ion chromatograms in Fig. 4 also furnish GC elution temperatures for formyl-Met and Leu-Phe, from DCP-digested fMLF, and thus allow comparison to the elution temperatures for putative formyl-Met and Leu-Phe from DCP-treated fraction 23. GC elution temperatures further confirm the identity of the compounds as obtained from mass spectra. As Fig. 4 shows, there is a match of elution temperatures for formyl-Met and Leu-Phe between DCP-treated fMLF and fraction 23. (See below for mass spectral data.)

Fig. 5 shows the computer-generated total ion (A1 and B1) and selected ion (A2, A3, B2, and B3) plots from the two GC-MS runs shown in Fig. 4. The selected ion plot allows one to pick out materials exhibiting a particular ion in their mass spectra, thus simplifying searches of the computer stored data for a given possible mass spectrum. The total ion plots allow reference back to the original total ion chromatograms for such purposes as determining the GC elution temperature of a particular peak. Here, the selected ion mass of 247 is used to search for TMS formyl-Met, and the ion mass of 158 is used to search for TMS Leu-Phe. The ion mass of 247 results from loss of CH₃SCH=CH₂ from the molecular ion of TMS formyl-Met, and the ion mass of 158 corresponds to the ion [TMS—NH=CHCH₂CH(CH₃)₂]+. The selected ion technique was also used to search for the presence of Phe in the GC-MS analysis of DCP-digested fraction 23 and, as would be expected from the digestion of authentic fMLF, Phe was also observed in this case. When this technique was further employed to search for dipeptides, no dipeptides other than Leu-Phe were found. One peak from each of the two selected ion traces from the GC-MS analysis of DCP-treated fraction 23 matched with the correct GC retention temperatures for formyl-Met and Leu-Phe. The other 4 fractions functioned as excellent blank controls, as similar selected ion traces did not show peaks at points where formyl-Met and Leu-Phe eluted from the GC.

Fig. 6 shows the mass spectra of TMS formyl-Met and Leu-Phe as obtained from GC-MS analysis of either DCP-treated fMLF or HPLC fraction 23, which are exhibited in A1 and B1 and A2 and B2, respectively. In each spectrum, the ion used in the selected ion plot to locate the spectrum is labeled. Also labeled is the molecular weight-determining ion [M-15]+ for each compound. The facile loss of 15 mass units (CH₃) from the molecular ion is typical for TMS compounds. Because of sample contamination by siloxanes from HPLC column bleed, the mass spectra of TMS formyl-Met and Leu-Phe from DCP-treated fraction 23 have extraneous peaks, such as those at m/e 217, 295, or 355, or peaks of greater than expected intensity, such as those at m/e 73 or 147. In the case of TMS formyl-Met, there is a rather good match between the two spectra, in spite of this background. In the case of TMS Leu-Phe, where the background is higher, thus making the comparison somewhat more difficult, the presence of TMS Leu-Phe is readily confirmed by the presence of ion masses at m/e 158 and 407. By reference back through the computer-generated selected ion plot and total ion plot to the total ion chromatogram, it can be determined that the two materials exhibiting these mass spectra also have GC elution temperatures identical to those of authentic formyl-Met and Leu-Phe, respectively.

**HPLC Fractionation of Partially Purified E. coli Culture Filtrate Spiked with Formyl-Met-Leu-Phe**

To confirm further that peak 3 contained fMet-Leu-Phe and also to determine that the heterogeneous mixture of
peptides and butanol solvent in the crude extract would not alter the retention time of fMet-Leu-Phe, a preparation of partially purified *E. coli* filtrate with 2.5 nmol of added fMet-Leu-Phe was subjected to HPLC fractionation under identical conditions. Fig. 7 demonstrates that the retention time of fMet-Leu-Phe is not altered when applied to the column as a mixture with the crude extract. The synthetic fMet-Leu-Phe is eluted in the identical HPLC fraction (i.e. 45 min) as the active peak 3 material from the unspiked culture filtrate. In addition, only peak 3 showed an increase in the titer of the

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FIG. 5. Computer-generated total ion and selected ion plots from GC-MS analysis of DCP-treated fMLF (A1, A2, and A3) and HPLC fraction 23 (B1, B2, and B3). Row 1, total ion plot for each; Row 2, selected ion plot for m/e 247 used to search for formyl-Met; Row 3, selected ion plot for m/e 158, used to search for Leu-Phe.
Fig. 6. Mass spectra of TMS formyl-Met and Leu-Phe from GC-MS analysis of DCP-treated IMLF (A1 and A2, respectively) and HPLC fraction 23 (B1 and B2, respectively).
enzyme-releasing activity equivalent to the added fMLF, when the individual HPLC fractions were analyzed by bioassay (data not shown).

DISCUSSION

We have previously demonstrated that E. coli culture filtrates contain heat-stable chemotactically active peptides that range in size from 150 to 1500 daltons, and are amionic at neutral pH (3). The peptide nature of these chemoeffector substances has been established by various enzyme and chemical treatments. Chemotactic activity, for example, is sensitive to Pronase and carboxypeptidase A digestion. Resistance to leucine aminopeptidase treatment, dansylation, and to fluorescamine and ninhydrin treatments suggests that the NH₂-terminal position of the peptide is either blocked and/or not essential for the expression of activity. The behavior of the material on ion exchange resins also supports these data. In contrast, free carboxyl groups appear to be required for activity, since methyl esterification markedly depressed chemotactic activity. The absence of an appreciable effect by periodate treatment further suggests that carbohydrate is not essential for activity. Complete characterization of the bacterial chemotactic factor has been hampered by the minute concentrations of these active substrates; however, a purified preparation was reported to contain aspartic acid, serine, glutamic acid, alanine, and glycine (3). These amino acids are more hydrophilic than the amino acids we now report and are probably present in higher concentrations as suggested by these previous data using less sensitive techniques. The majority of peptides containing these amino acids probably would correspond to the early peaks in the HPLC fractionation (i.e. 20% CH₃CN) and thus were not resolved for the bulk of the UV-detectable mass (Fig. 2), which is devoid of biological or antigenic activity (Fig. 3. A and B).

It is also apparent from this earlier work that there is considerable heterogeneity in the bacterial chemotactic factors. Depending on the isolation and purification procedure involved, three to five separate chemotactic activities are resolved (3). These extensive studies concluded that E. coli organisms elaborate in very low concentrations numerous peptides of similar composition, which may differ markedly in their specific chemotactic activities. Of interest is that one of the major activities separated by Sephadex G-15 gel filtration had an estimated molecular weight of 440, which corresponds closely to the M₇, (437) of fMet-Leu-Phe (3).

The formyl peptide character of these attractants has been suspected since the recognition that a large number of bacteria elaborate similar chemotactic substances (1, 2). Formyl methionyl peptides are derived from the NH₂-terminal regions of newly synthesized proteins. With some proteins, including those found in the cytoplasm, the formyl methionyl peptides are cleaved post-translationally and are not found in the mature protein. In the case of membrane and secretory proteins which possess NH₂-terminal signal peptides, these NH₂-terminal extensions are cleaved by a signal peptidase following appropriate polypeptide transport and, therefore, could be released into the extracellular fluid surrounding the cell membrane (21). The signal peptide is encoded in the form of a short-lived sequence extension and is believed to direct the transport of newly synthesized polypeptides across the appropriate membrane following their synthesis (21). The release of these prokaryotic formyl peptides could be, in part, the mediators responsible for leukocyte accumulation at foci of bacterial infections. These observations led to the discovery by Schifman et al. (4) that simple synthetic formylmethionyl peptides are chemotactic for leukocytes, whereas the nonformylated parent are not. Bennett et al. (22) have demonstrated using a cell-free coupled transcription-translation system that the precursor form of Bacillus licheniformis penicillinase (which contains the signal peptide) will stimulate lysosomal enzyme secretion by neutrophils, whereas the mature forms of the protein are completely inactive. The systematic studies by Showell et al. (8) and Freer et al. (19, 20) on the structure-activity relationship of various synthetic formylpeptides as chemotactic factors and inducers of lysosomal enzyme secretion suggested that their effect was mediated through binding to a stereoselective receptor on the rabbit neutrophil plasma membrane. The synthesis of radioabeled formylpeptide attractants has since allowed a specific receptor to be demonstrated on neutrophils and macrophages of several species (5, 10-15, 23). More recently, the potent spasmyogenic activity of the formyl peptides for guinea pig ileum has been shown to be mediated through binding to a similar receptor (24).

Additional evidence for the similarity in the structure of the bacterial chemotactic factor to the synthetic chemotactic peptides has come from both receptor-binding data and immunochemical data. With the former, the butanol extracts of E. coli culture filtrate used in our analysis inhibited specific binding of fMet-Leu-[¹⁴C]Phe to the neutrophil receptor at concentrations required to give a good chemotactic response (5).
We have also previously demonstrated by radioimmunoassay and bioassay that anti-fMet-Leu-Phe binds and removes the biologically active component of chemotactic factor-enriched extracts from *E. coli* culture filtrates (6). Furthermore, the N-formyl group is mandatory for maximal antibody-binding activity. Structurally related formylmethionyl peptides, with subtle changes in their amino acid composition and relative position, bind to the antibody with varying degrees of affinity.

In our present work, we have further extended the radioimmunoassay analysis to allow us to determine the extent of separation and to quantitate recovery of antigenic activities by HPLC. Three peaks of activity were found (Fig. 3B), and the largest antigenic peak (Fraction 23) was found to contain 66% of the recovered antigenic activity and corresponded exactly to the major peak of biological activity in which an equivalent amount of enzyme-releasing activity was found (Table I). Further analysis of HPLC peak 23 by DCP/GC-MS methodology demonstrated the presence of formyl-Met-Leu-Phe in this fraction. This was done via detection of formyl-Met and Leu-Phe, which arise from fMLF via a single cleavage at the Met-Leu bond by DCP. Additional confidence in this analysis was gained from mass spectral and GC elution data obtained with authentic fMLF. Because of the presence of artifactual contaminants that accumulated during isolation of fMLF, such as siloxanes from HPLC column bleed, other mass spectral methodologies that use direct, rather than GC introduction into the MS to analyze for this compound, such as fast atom bombardment MS are not as suitable, because the ions identifying fMLF would be obscured by ions arising from these contaminants. Also, as can be qualitatively deduced from the peak heights in the total ion chromatogram, the amount of fMLF present is only in the range of about 5 pmol, an amount that is too low to normally be analysed by other MS approaches at their current state of technical development.

The lack of a detectable formyl-methionyl peptide in HPLC peaks 1 and 5 using the DCP/GC-MS methodology, despite the presence of substantial antigenic activity and UV-detectable material, could arise from several possibilities. One of these is that these materials are peptides with an even number of amino acids, which upon DCP digestion, would yield a formyl-Met-X dipeptide, which, depending on X, might not elute from the GC. A second possibility is that these materials cannot be degraded by DCP because of a blocked COOH terminus or an unfavorable conformation. Another possibility is that these materials may be present in amounts below the limits of detection using this methodology. Thus no definitive identification of these other putative peptides can be made at this time.

Our inability to detect antigenic activity in peaks 2 and 4 despite strong enzyme-releasing activity in these same fractions may have several possible explanations. First, and most likely, these activities may represent extremely active formyl peptides which contain at least 4 amino acids. This is based on the structural evidence that addition of a hydrophobic 4th amino acid to the tripeptide fMet-Leu-Phe (the most active tripeptide synthesized thus far, (8, 19, 20)) will increase enzyme-releasing activity 20- to 100-fold. Thus substantial biological activity may be seen where inadequate mass is available to detect by radioimmunoassay (i.e. anti-fMet-Leu-Phe has minimal recognition of any amino acid beyond position 3 (6)). This same explanation would explain our inability to detect any formylmethionyl peptides by DCP/GC-MS. A second possible explanation is that these activities are associated with peptides structurally unrelated to the formyl peptides.

The finding that fMet-Leu-Phe is a naturally occurring peptide provides confirmation of the link between studies using the analogue synthesized almost a decade ago (8) and the naturally occurring bacterial factors. Thus, the formyl peptides will continue to provide an elegant and valid model system for the study of the many complex biological responses exhibited by phagocytic cells. Their importance physiologically in triggering an acute inflammatory response in tissue-containing bacteria by directing leukocyte migration toward the invading microorganisms is apparent. The same peptides also possess potent smooth muscle-contracting activity, a response seen with other acute inflammatory mediators (24).

Although we present evidence that as few as 3 and as many as 5 activities can be separated and identified in bacterial culture filtrates, of which fMet-Leu-Phe is the major component, the precise cellular origin of these peptides remains unknown. Whether the fMet-Leu-Phe leading sequence is unique to a particular bacterial strain, to a single signal peptide, or is common to a particular class of proteins (i.e. cytoplasmic proteins, proteins bound for secretion or assembly in the outer membrane) remains to be determined. However, in the almost 30 signal peptides that have now been sequenced the fMet-Leu-Phe sequence has not been found (25). Indeed, with the rapid advancement in the studies of prokaryotic polypeptide structure and transport, these questions may soon be answered.

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Note Added in Proof—While this paper was in press, Miyake et al. (26) reported the isolation of two neutrophil chemotactic factors from culture filtrates of *Streptococcus sanguis*. These peptides were chemically characterized as NH₂-terminal blocked peptides and methionine was detected as the sole NH₂-terminal amino acid. Compositional analysis showed that one of the factors consisted of methionine, isoleucine, leucine, and phenylalanine and the other of proline, valine, methionine, isoleucine, and leucine.

REFERENCES
1. Ward, P. A., Lepow, I. H., and Newman, L. J. (1968) Am. J. Pathol. 52, 725-736
2. Keller, H. U., and Sorkin, E. (1967) Int. Arch. Allergy Appl. Immunol. 31, 505-517
3. Schiffmann, E., Showell, H. J., Corcoran, B. A., Ward, P. A., Smith, E., and Becker, E. L. (1975) J. Immunol. 114, 1831-1837
4. Schiffmann, E., Corcoran, B. A., and Wahl, S. M. (1976) Proc. Natl. Acad. Sci. U. S. A. 72, 1059-1062
5. Aswanikumar, S., Corcoran, B., Schiffmann, E., Day, A. R., Freer, R. J., Showell, H. J., Becker, E. L., and Pert, C. B. (1977) Biochem. Biophys. Res. Commun. 74, 810-817
6. Marasco, W. A., Showell, H. J., Freer, R. J., and Becker, E. L. (1982) J. Immunol. 128, 956-962
7. Marasco, W. A., and Becker, E. L. (1982) J. Immunol. 128, 963-968
8. Showell, H. J., Freer, R. J., Zigmund, S. H., Schiffmann, E., Aswanikumar, S., Corcoran, B. A., and Becker, E. L. (1976) J. Exp. Med. 145, 1154-1169
9. Marasco, W. A., Fantone, J. C., Freer, R. J., and Ward, P. A. (1983) Am. J. Pathol. 111, 273-281
10. Williams, L. T., Snyderman, R., Pike, M. C., and Lefkowitz, R. J. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 1204-1208
11. Chenoweth, D. E., Rowe, J. G., and Hugli, T. E. (1979) Fed. Proc. 38, 1454 (abstr.)
12. Snyderman, R., and Fuhrman, E. J. (1980) J. Immunol. 124, 2754-2757

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13. Snyderman, R., and Pike, M. C. (1980) Science 209, 493–495
14. Marasco, W. A., Niederhuber, J. E., and Ward, P. A. (1982) Fed. Proc. 41, 272 (abstr.)
15. Spilberg, I., Mehta, J., Daughaday, C., and Simchowitz, L. (1981) J. Lab. Clin. Med. 97, 602–609
16. Sharp Electronics Corp., Pocket Computer Applications Manual, PC-1500
17. Yarmush, M. L., Krutzsch, H. C., and Kindt, J. J. (1980) Mol. Immunol. 17, 319–326
18. Krutzsch, H. C. (1983) Methods Enzymol. 91, 511–524
19. Freer, R. J., Day, A. R., Radding, J. A., Schiffman, E., Aswani-umar, S., Showell, H. J., and Becker, E. L. (1980) Biochemistry 19, 2404–2410
20. Freer, R. J., Day, A. R., Muthukumaraswamy, N., Pinon, D., Wu, A., Showell, H. J., and Becker, E. L. (1982) Biochemistry 21, 257–263
21. Blobel, G., Walter, P., Chang, C. N., Goldman, B. M., Erickson, A. H., and Lingappa, V. R. (1979) Symp. Soc. Exp. Biol. 33, 9–36
22. Bennett, J. P., Hirth, K. P., Fuchs, E., Sarvas, M., and Warren, G. B. (1980) FEBS Lett. 116, 57–61
23. Weinberg, J. B., Muscato, J. J., and Niedel, J. E. (1981) J. Clin. Invest. 68, 621–630
24. Marasco, W. A., Fantone, J. C., and Ward, P. A. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 7470–7473
25. Perlman, D., and Halvorson, H. O. (1983) J. Mol. Biol. 167, 391–409
26. Miyake, Y., Yasuhara, T., Fukui, K., Suginaka, H., Nakajima, T., and Moriyama, T. (1983) Biochim. Biophys. Acta 758, 181–186
Purification and identification of formyl-methionyl-leucyl-phenylalanine as the major peptide neutrophil chemotactic factor produced by Escherichia coli. W A Marasco, S H Phan, H Krutzhc, H J Showell, D E Feltner, R Nairn, E L Becker and P A Ward

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