Diethyl Phthalate, a Chemotactic Factor Secreted by Helicobacter pylori*

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From the †The Bing Foundation (to P. A.), National Institutes of Health Grant K24 (Al 01610) (to P. A.), the Mucoosal Immunology Core Grant AI 28897 (to P. A.), the City of Hope National Institutes of Health Cancer Center Core Grant CA3572 (to D. A. K.), NIDDK, National Institutes of Health CURE Digestive Diseases Research Center Grant DK-41301 (to J. H. W. and J. R. R., Jr.), the Department of Veterans Affairs Research Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertised" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Many factors that cause chemotaxis of immune cells have been identified (1). For example, there are more than 30 structurally related 8- to 10-kDa proteins named chemokines (e.g. interleukin-8) that are potent immune cell chemoattractants (2). In addition, several arachidonic acid-derived lipids (e.g. leukotriene-B4 (3)) and the tripeptide formyl-Met-Leu-Phe (fMLP, 1 (4)) are known to be potent stimulators of monocyte and neutrophil chemotaxis. These chemotactic factors have been implicated in human disease states associated with inflammation such as gastritis (5, 6), rheumatoid arthritis (7), inflammatory bowel diseases (8, 9), and asthma (10). Chemotaxis may play a role in recruitment of inflammatory cells during infection by Helicobacter pylori leading to gastritis and peptic ulcerations (11). H. pylori infection is now accepted as the major cause of gastroduodenal disease (12) and is associated with increased risk for development of gastric carcinoma (13–15) and primary non-Hodgkin’s lymphomas of the stomach (16). Although the infection is widespread in the human population most infected people have no symptoms of disease, perhaps because of the genetic diversity of H. pylori strains (17) and/or individual differences in susceptibility.

Identifying the specific bacterial agents that lead to the mucosal inflammatory response is important for understanding the role of H. pylori in gastroduodenal disease. The three possible mechanisms suggested by Crabtree (18) for the mucosa inflammatory response generated by H. pylori infection are: 1) a direct stimulation of monocyte and neutrophil chemotaxis to the gastric epithelial cells via factors released from the bacteria, 2) an indirect stimulation of gastric epithelial cells by H. pylori factors to release chemotactic agents, and 3) an indirect activation of immune cells by H. pylori factors to release chemotactic agents. These mechanisms are supported by evidence that this essentially non-invasive bacterium can cause neutrophil infiltration at a distance. In a gerbil model of gastritis, the initial damage caused by H. pylori infection was due to colonization of the antrum with subsequent mucosal lesions in the fundus (19). In a rat model, bacteria-free filtrates caused delayed healing of pre-existing ulcers with active chronic inflammation (20). Furthermore, other work shows that the bacteria are rarely found within the mucosa and mostly reside in the mucus layer overlaying the gastric epithelium (21). Thus the factor(s) that cause gastric inflammation presumably diffuse through the mucus and through the gastric epithelium to indirectly or directly generate the immune cell migration.
These observations led others to investigate factors produced by *H. pylori* that cause monocyte and neutrophil chemotaxis (19, 22–31). Specific factors identified include: *H. pylori* sonicate proteins of 25–35 kDa (24, 25), urease and urease fragments (26), leukotrienes (22), fMLP (5), an 8.5-kDa factor (28), a 10.5-kDa factor (29), a 30-kDa porin protein (31), and a low molecular weight factor that is not fMLP (27, 30).

In this work the low molecular weight chemotactic factor from *H. pylori* supernatants has been identified as diethyl phthalate (DEP).

**MATERIALS AND METHODS**

**Bacterial Strains, Culture Conditions, and Supernatant Collection—** The *H. pylori* strain ATCC 43579 (American Tissue Cell Culture) was grown under microaerophilic conditions at 37 °C in bruccella broth (Difco Laboratories, Detroit, MI) with 1% heat-inactivated PBS, 10 μg/ml vancomycin (Sigma), and 2.5 units/ml polymyxin B (Sigma) for 48 to 72 h. The bacteria were harvested during log growth phase. The resultant cultures were centrifuged at 3000 rpm, and the pellet washed twice with calcium- and magnesium-free PBS. The final pellet was then re-suspended with PBS, which was pre-screened for chemotactic activity, to give an absorbance value of 0.9 units at 620 nm (10^10 colony-forming units/ml). The re-suspended *H. pylori* were incubated in PBS for 4 h at 37 °C and harvested again by centrifugation at 3000 rpm for 30 min at 25 °C. The supernatant was then filtered through a 0.22-mm filter and either used immediately or stored at −80 °C. Frozen supernatants could be thawed and show chemotactic activity for up to two months after collection.

**Isolation of Monocytes and the Chemotaxis Assay—** Monocytes were isolated from the peripheral venous blood of healthy donors on a Ficoll-Hypaque density gradient (Histopaque, Sigma). The cells were washed and re-suspended in Dulbecco’s modified Eagle’s medium Low Glucose (Invitrogen) containing 1% heat-inactivated PBS. Cells were confirmed as monocytes by microscopy using Wright stain and counted using a hemocytometer. The monocyte concentration was diluted to a final concentration of 10^5 cells/ml.

The chemokinetics of monocytes in response to *H. pylori* supernatant fractions was essayed using 48-well chemotactic chambers (NeuroProbe, Inc., Cabin John, MD) (32). The upper chamber was separated from the lower chamber by a polyvinyl propylene-free nitrocellulose filter with a 5 μm pore size (Costar, Pleasanton, CA). The upper compartment of the chemotactic chamber was loaded with 50 μl of the mononuclear cell suspension. The lower compartment was dilutions of the sample to be tested. Control wells containing 10^-8 M fMLP (positive control) or PBS (negative control) were run in each assay. Following an incubation period of 1.5 h at 37 °C, the filters were stained with Giesma stain, and the number of monocytes that had migrated to the lower surface of the filter was counted microscopically and tallied as the mean of 20 random high-power fields for each sample. This assay was done at least three times. The cells counted were expressed as the mean number of monocytes per high-powered field. The chemokinetics activity (or cell movement unrelated to the concentration gradient) was distinguished from chemotactic activity (directed cell movement) by checkerboard analysis. The checkerboard analysis consists of serial dilutions of the test sample placed in the lower compartment of the chamber and the same serial dilutions of the monocytes in the upper compartment. Directional movement of the cells in response to a concentration gradient was graded as chemotactic activity, whereas increased movement of cells unrelated to the concentration gradient was defined as chemokinetics.

**Chemotactic Activity Purification—** Conditioned supernatants with chemotactic activity were concentrated by solid phase chromatography on SepPak™ cartridges (Waters-Millipore, Milford, MA). Five “classic” cartridges containing 360 mg of C-18 packing material were connected in series. The cartridges were pre-rinsed with 50 ml of ethanol followed by 50 ml of an aqueous 0.1% acetic acid solution. One liter of absorbing material were collected manually into polypropylene tubes. The active fraction from the 70% ethanol eluant was diluted 5-fold with 0.1% trifluoroacetic acid then loaded onto a reverse phase HPLC column (Vydac C-18 reverse phase HPLC column, 4.6 × 250 mm, 5 μ, catalog number 218TP54, Separation Group, Hesperia, CA) equilibrated with 0.1% trifluoroacetic acid. Samples were loaded in 4-ml increments through a 5-ml sample injector. The column was rinsed for 5 min. with this 0.1% trifluoroacetic acid, then eluted with a rapid (10 min) gradient to 25% acetonitrile and a slow gradient (60 min) to 40% acetonitrile. The effluent was monitored at 220 and 280 nm, and fractions were collected manually.

**Preparation of SepPak™ and Reverse Phase HPLC Fractions for Bioassay—** A portion (5–10%) of each fraction was diluted 5-fold with 0.1% acetic acid, loaded onto a single SepPak™ cartridge that was pre-rinsed with 10 ml of ethanol, followed by 10 ml of 0.1% acetic acid. After loading the cartridge was rinsed with 0.1% acetic acid and eluted with 1.5 ml of 70% ethanol containing 0.1% acetic acid. HPLC column controls containing 35% acetonitrile were diluted, loaded, and eluted in the same manner and were shown to have no chemotactic activity. The water/acetic acid/ethanol buffer caused no interference in the chemotactic assay.

**Preparation of the Purified Chemotactic Agent for NMR—** To measure the ^1H-NMR signals of the chemotactic factor eluted in a protonated water/ethanol/acetic acid solvent system it was necessary to exchange this solution for one containing deuterated solvents. The buffer exchange was accomplished using a micro-capillary HPLC system designed and built at the Beckman Research Institute of the City of Hope. In brief, an ISCO100DM syringe pump was used to deliver a 0.1% trifluoroacetic acid solution under constant pressure control to displace a preformed reverse phase gradient from a six port switching valve (Rheodyne Model 7000, Cotati, CA) directly onto a 0.5-cm bonded silica gel column constructed and packed as previously described (33). The sample injector (Rheodyne Model 7125) was placed upstream relative to the gradient valve to minimize the dead volume between the gradient loop and column. The deuterated organic gradient was produced off-line using two Harvard Apparatus Model 44 programmable syringe pumps and a reduced volume tee connector for mixing and storing the gradient in a loop. The gradient loop was back-filled in reverse order to place the start of the gradient at the front of the loop. The gradient was formed while the sample was being loaded onto the column and was switched on-line following the completion of the loading step.

The micro-column effluent was monitored at 200 nm using an ABI model 757 UV visible absorption spectrometry detector equipped with a capillary flow-cell holder. The detector output was recorded on a Soltec model 1241 strip-chart recorder. A steep linear gradient from 5 to 95% buffer B (Buffer A, 0.1% trifluoroacetic acid in D_2O; Buffer B, deuterated acetonitrile-CD_3CN) over 5 min at a flow-rate of 20 μl/min was used to elute the chemotactic activity. Fractions containing the 200 nm of absorbing material were collected manually into polypropylene tubes. The active fractions were pooled and re-dissolved in the aqueous solution containing trifluoroacetic acid and CD_3CN. The trifluoroacetic acid remained in the aqueous phase while the chemotactic factor and CD_3CN were extracted into the methylene chloride solvent.

**NMR—** The NMR experiments on the purified chemotactic agent were performed on a Varian Unity Plus NMR spectrometer with a 500 MHz proton frequency, and the probe air temperature regulated at 25 °C. A 700-μl aliquot of the pooled, CD_3CN extracted sample described above was placed in an NMR tube and sealed with Parafilm™. Typical NMR parameters were: an acquisition time of 1.5 s, a 6000-Hz spectral width, 128–256 transients co-added with a 10-s delay between each transient, a pre-saturation delay of 1 s at the frequency of the H_2O signal (2.5 ppm), and the transmitter channel used for both acquisition and decoupling. Synthetic DEP was used as received with a 5-μl of (5.6 mg) aliquot diluted in 1 gram of CD_3CN (~25 mM DEP), and this sample sealed in an NMR tube. This solution was also used to spike the NMR sample derived from the *H. pylori* supernatant to demonstrate signal superposition.**

**GC/MS of the Purified Chemotactic Agent and Synthetic DEP—** Two different GC/MS Instruments were used. HPLC fractions containing the purified chemotactic agent were dried in a stream of nitrogen and re-dissolved in 10 μl of ethyl acetate, and 1–3 μl of aliquots were loaded onto a solvent-free GC injector (dripping needle type, Ray Allen Assoc., Boulder, CO) connected to a bonded phase fused silica capillary column (DB-1H, 15 μm, 0.26 mm inner diameter, film thickness 0.25 microns, J & W Scientific, Folsom, CA) using helium as the carrier gas. The end of the fiber was inserted into the ion source of a modified HP 5985B GC/MS instrument. A head pressure of 1.5 p.s.i. of helium was maintained in the GC injector port. The injector port and transfer line were maintained at 250 °C. The GC oven was held at 80 °C for 1 min following injection and then increased linearly at 10 °C/min to a plateau of 300 °C. The mass spectrometer was operated in the electron ionization mode with a high-energy dynode detector (Phrasor Scientific, Du-

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arte, CA) set at −5 kV. The MS ion source was held at 200 °C, and an ion current of 300 microamps (70 electron volts) was used. Solutions of authentic standards including methyl stearate, benzophenone, and DEP were analyzed under identical conditions.

SepPak™ purified samples from control experiments designed to test the possibility that DEHP was converted to DEP during sample preparation were concentrated in stream of dry nitrogen, then redissolved in dichloromethane, and injected with a Grob-type splitless injector onto a bonded phase fused silica capillary column (DB-SMS, 30 m, 0.25 mm inner diameter, film thickness 0.25 microns, J & W Scientific, Folsom, CA) using helium as the carrier gas. The end of the column was inserted directly into the ion source of a Finnigan 4000 GC/MS instrument. A head pressure of 11 p.s.i. of helium was maintained in the GC injector port. The injector port and transfer line were maintained at 280 °C. The GC oven was held at 30 °C for 4 min following injection and then increased linearly at 6 °C/min to a plateau of 300 °C. The mass spectrometer was operated in the electron ionization mode. The MS ion source was held at 240 °C, and an ion current of 500 microamps (70 electron volts) was used.

HPLC Co-chromatography of Purified and Synthetic Diethyl Phthalate—Preliminary results showed that synthetic DEP and purified chemotactic factor eluted at 31.9 and 31.4 min, respectively, during elution on a C-18 reverse phase column. Equal peak areas of absorbance at 220 nm of DEP (25 μl of neat DEP diluted 1,000-fold in ethanol and then 10-fold in 0.1% trifluoroacetic acid) and the purified chemotactic factor (25 μl from final purification step) were co-injected and eluted under the same conditions to determine whether they eluted as a single peak.

Control Incubations to Determine the Source of DEP—Phthalate esters are used as plasticizers in the manufacture of polyvinyl chloride. All manufacturers of plasticware and filters used in this study were contacted, and they stated that no phthalates were used in the manufacture of their plasticware. However, it was felt that it was important to verify that the purified chemotactic agent actually came from bacteria and not from outside sources. To verify that the source of the chemotactic agent was not from the plasticware the same series of procedures were performed on the media without bacteria present, including: incubation of the brucella broth, the SepPak™ concentration step, solution volumes of incubations, and the type of glassware and plasticware used (these experiments were performed in parallel with the experiments on media containing bacteria). The concentrates from cultures with and without bacteria were examined by reverse phase chromatography (see Fig. 6 legend) for the presence of the chemotactic agent. The absorbance at 220 and 280 nm were monitored, and fractions were collected and assayed for chemotactic activity. As an additional control, a solution of synthetic di-(2-ethylhexyl)phthalate (DEHP, 0.1 mg/2 ml) was loaded on the SepPak™ column and eluted with ethanol as described above. This solution was diluted and tested for chemotactic activity.

RESULTS

Chemotactic Assay—Monocytes migrated toward concentrated supernatants from H. pylori incubated for 1.5 h in physiological saline (Fig. 1) in a dose-dependent manner. The peak chemotactic activity from the purified supernatant was ~70% the value observed for a 10−8 M solution of fMLP (positive control) (Fig. 2). During the purification of chemotactic agent it was determined that acetoniitriile/trifluoroacetate HPLC buffers interfered in the chemotactic assay. Therefore, a buffer exchange was developed with a 0.05–0.1 M aliquot of the HPLC fractions that exchanged the HPLC buffer for an acetic acid/ethanol buffer on a SepPak™ cartridge before the chemotactic assay. The acetic acid/ethanol buffer caused no interference in the chemotactic assay.

Purification of the Chemotactic Factor from H. pylori Supernatants—H. pylori cultured for 4 h in physiological saline gave a slightly turbid solution. This was concentrated on a SepPak™ solid phase chromatography. A partial purification was achieved by first eluting the SepPak™ with 30% ethanol (this contained no chemotactic activity) followed by another elution with 70% ethanol. The 70% ethanol eluant was diluted with 0.1% trifluoroacetate loaded on a C-18 reverse phase HPLC column and eluted with an increasing gradient of acetonitrile. The active fractions from two separate purifications were pooled, diluted with 0.1% trifluoroacetate loaded onto the same column and eluted in the same manner as the first HPLC step. One major absorbance peak eluted (Fig. 2A) with absorbance at 220 nm and 280 nm (profile for 280 nm not shown). This peak contained chemotactic activity while adjoining fractions did not (Fig. 2B).

Preparation of the Purified Chemotactic Agent for NMR—A micro-scale solvent exchange system was used to prepare the NMR samples because the intensity of CH3CN signal in the un-modified sample obscured the much smaller signals present from the purified chemotactic agent. The HPLC-purified sample described above was subjected to a micro-column HPLC procedure to replace the protonated acetonitrile with deuterated acetonitrile. A greatly reduced signal from protonated CH3CN at (1.94 ppm) resulted from this procedure.

NMR of the Purified Chemotactic Agent and Synthetic DEP—The chemical shifts and integrated areas of the NMR signals are consistent with DEP. The three major signals aside from solvent peaks were found centered at 7.571, 4.234 (quartet), and 1.250 (triplet) ppm (shown in the expanded sections of Fig. 3). The relative ratio of integrals for these signals is 2:2:3, respectively. The symmetric set of aromatic signals at 7.63 and 7.52 ppm are consistent with an R,R′ ortho-substituted aromatic ring. The signals at 4.234 and 1.250 are spin-coupled to each other and have the chemical shift appropriate for an aromatic bound ethoxy group. The signal at 1.2 ppm is apparently from an impurity.

Signals from residual protonated methylene chloride, acetoniitriile and water were present in the NMR spectrum. The signals from the protonated acetonitrile were reduced by solvent exchange, and the CH3Cl2 signal were small enough so that they did not interfere with signals from the purified chemotactic factor. In addition, the signal from the water remaining after the methylene chloride extraction was reduced by presaturation. The other smaller signals observed between 0.8 and 4.0 ppm are not part of DEP because of their smaller intensities relative to DEP signals and because any other substitution pattern of the benzene ring would greatly alter the coupling patterns of the molecule.

The NMR spectra acquired with synthetic DEP shows that the purified chemotactic agent is diethyl phthalate. The same pattern of signals with the same relative areas is observed for synthetic DEP as seen in Fig. 3 (data not shown). The chemical shifts of synthetic DEP in 99.99% CD3Cl2 (7.628, 4.328, and 1.347 ppm for aromatic, methylene, and methyl signals) are slightly altered from those observed in the sample derived from...
the bacteria. These small changes are likely due to the presence of acetonitrile in the purified sample from *H. pylori* supernatant. This was confirmed by spiking the purified sample with synthetic DEP and observing increases in the intensities of only signals assigned to DEP.

**GC/MS of Purified Diethyl Phthalate**—Aliquots of the HPLC-purified chemotactic factor and synthetic DEP were analyzed by combined gas chromatography and mass spectrometry. The purified chemotactic factor and DEP had indistinguishable GC retention times (6.02 and 6.04 min, respectively). Under electron ionization conditions the purified compound gave three main ions at m/z 222, 177, and 149 corresponding to the molecular ion and two fragment ions. The mass spectrum of the purified chemotactic agent is represented by the top tracing, and that of synthetic DEP by the inverted lower tracing. The two spectra both show prominent ions at m/z 222, 177, and 149 corresponding to the molecular ion and two fragment ions. The results indicate that the chemotactic activity purified from *H. pylori* supernatants is indistinguishable from DEP.

**Control Incubations to Determine the Source of DEP**—To confirm the source of DEP a sequential subtraction assay was performed. All glassware and buffers were incubated without *H. pylori* and processed in an identical manner (e.g. solution volumes, times of incubations, and the type of glassware and plastic were the same). Fig. 6 shows that the two control incubations without *H. pylori* present contained no detectable amount of DEP, while the two incubations with *H. pylori* present had significant amounts of diethyl phthalate. The manufacturers of all the plasticware (Costar, Becton Dickinson, Nalgene, Sherwood Medical Company, Rainin, and S&S) and the filter (Neuro-probe) used in this work were contacted and none of them claimed to use DEP, (DEHP), or any other phthalate in the production of the plasticware.

Further controls performed with synthetic DEHP verified that DEHP from an unknown source was not a precursor of DEP in the absence of *H. pylori*. Specifically, DEHP could produce DEP by transesterification of both (ethyl)hexyl groups in a reaction with the ethanol used as an eluant in the SepPak® purification step. Therefore a dilute solution of DEHP was treated in the same manner as culture media to determine whether it was converted into a chemotactic factor in the absence of bacteria. This solution exhibited no chemotactic activity in the monocyte chemotaxis assay described above (data not shown). Furthermore GC/MS analysis of the eluants of DEHP from the SepPak® showed only DEHP; no DEP was present. These results do not exclude the possibility that *H. pylori* take DEHP and produces DEP. Even in this case the DEP is bacterially derived.

**Synthetic Diethyl Phthalate Causes Chemotaxis**—The effect of synthetic diethyl phthalate on migration of monocytes was
profile of 1 liter of supernatant from *H. pylori* respectively, bacteria present. The upper panels concentration on SepPaks™. The UV visible absorption spectrometry, GC and HPLC retention on the basis of multiple criteria including nuclear magnetic factor has been shown to be indistinguishable from that of DEP reverse phase HPLC steps. The structure of this chemotactic up of bacterial supernatants followed by a two high-resolution column and eluted as described under *Materials and Methods*. The purified chemotactic factor were injected onto a reverse phase HPLC agent and synthetic DEP. The purified chemotactic factor is indistinguishable from synthetic DEP by activity.

In this work, a small-molecule, non-peptide chemotactic factor has been purified to virtual homogeneity from *H. pylori* supernatants. A and B represent separate incubations with and without, respectively, bacteria present. The upper panels show the absorbance profile of 1 liter of supernatant from *H. pylori* suspension cultures after concentration on SepPaks™. The lower panels show the one liter of culture media incubated for the same duration as the upper panels without bacteria. The arrows show the established elution position of synthetic DEP, and the shaded areas the regions of chemotactic activity.

**FIG. 6.** Reverse phase HPLC elution profile of *H. pylori* supernatants. A and B represent separate incubations with and without, respectively, bacteria present. The upper panels show the absorbance profile of 1 liter of supernatant from *H. pylori* suspension cultures after concentration on SepPaks™. The lower panels show the one liter of culture media incubated for the same duration as the upper panels without bacteria. The arrows show the established elution position of synthetic DEP, and the shaded areas the regions of chemotactic activity.

**DISCUSSION**

In this work, a small-molecule, non-peptide chemotactic factor has been purified to virtual homogeneity from *H. pylori* supernatants based on its ability to cause monocyte chemotaxis. Purification was achieved with a single SepPaks™ clean up of bacterial supernatants followed by a two high-resolution reverse phase HPLC steps. The structure of this chemotactic factor has been shown to be indistinguishable from that of DEP on the basis of multiple criteria including nuclear magnetic resonance spectroscopy, electron impact mass spectrometry, UV visible absorption spectrometry, GC and HPLC retention compared with fMLP. Synthetic DEP was dissolved in ethanol at a 0.1 mM concentration. This stock solution was diluted with PBS for measurement of chemotactic activity. The activity of DEP was compared with PBS controls and $10^{-8}$ M fMLP in the chemotactic assay. The maximum migration caused by 1.4 $\mu$M DEP was ~70% of that observed for fMLP (Fig. 7). The purified DEP concentration is estimated to be ~1.5 $\mu$M based on the comparison of high field view values obtained for the purified (Fig. 2) and synthetic DEP (Fig. 7) relative to fMLP.

The number of monocytes chemotaxing to various doses of DEP (1.25–5 $\mu$M) is determined per high power field (HPF) (solid bars); PBS (empty) and fMLP (crosshatched) are controls. Values are ± S.E.

**FIG. 7.** DEP-stimulated chemotaxis of monocytes. The number of monocytes chemotaxing to various doses of DEP (1.25–5 $\mu$M) is determined per high power field (HPF) (solid bars); PBS (empty) and fMLP (crosshatched) are controls. Values are ± S.E.

A toxin that has been identified as a polyketide-derived 12-membered ring macroline called mycolactone (34). *M. ulcerans* is the causative agent of Buruli ulcer where, similar to *H. pylori*, the necrosis caused by the bacteria extends some distance from the site of bacterial colonization. In another example, 6-methylsalicylic acid was produced by polyketide biosynthesis in *Penicillium olsonii* and transfected *Streptomyces coelicolor* (35, 36). Fungal production of di-(2-ethylhexyl)phthalate has been reported from *Penicillium olsonii* (37).

Only one chemotactic activity was observed in our HPLC elution fractions in amounts that could be detected by the chemotaxis assay. The 25–35-kDa sonicate proteins, urease and urease fragments, leukotrienes, 30-kDa porin protein, 10.5- and 8.5-kDa factors, and fMLP (5, 22, 24–30) are probably produced in different amounts than DEP and have very different size, charge, and hydrophobic character from DEP. Thus, the absence of these factors in our purification could indicate that these factors were not present in sufficient amounts in the strain of *H. pylori* used to cause monocyte migration or that differences in the methods used in sample preparation, purification, and assay caused selective detection of DEP over the other chemotactic factors.

**FIG. 5.** HPLC co-chromatography of the purified chemotactic agent and synthetic DEP. Equal peak areas of synthetic DEP and the purified chemotactic factor were injected onto a reverse phase HPLC column and eluted as described under “Materials and Methods.” The purified chemotactic factor is indistinguishable from synthetic DEP by reverse phase HPLC.

Times, and activity toward monocytes. Control experiments have unequivocally confirmed that purified DEP is not derived from incubation or preparation materials, confirming the statements obtained from the manufacturers of the plasticware used in these studies that claimed no phthalate were present. Therefore we have established that DEP is produced by *H. pylori* and propose that it represents a new class of chemotactic factor.

Our demonstration that DEP is produced by *H. pylori* is the first example to our knowledge that a phthalate ester is produced by a bacterium. Other bacteria and fungi have been shown to be capable of producing small molecular weight non-peptide factors. For example, *Mycobacterium ulcerans* secretes a toxin that has been identified as a polyketide-derived 12-membered ring macrolide called mycolactone (34). *M. ulcerans* is the causative agent of Buruli ulcer where, similar to *H. pylori*, the necrosis caused by the bacteria extends some distance from the site of bacterial colonization. In another example, 6-methylsalicylic acid was produced by polyketide biosynthesis in *Penicillium olsonii* and transfected *Streptomyces coelicolor* (35, 36). Fungal production of di-(2-ethylhexyl)phthalate has been reported from *Penicillium olsonii* (37).

Recent data support the possibility that DEP is a chemotactic factor in humans (38, 39). Seven monoeaster metabolites of...
phthalates that are used in plastics were measured in urine samples from a reference population of 289 adult humans. The DEP monoester was found in the highest concentration (range 0.12–16.9 \mu M, 5th and 95th percentile, respectively) with a mean value of 1.55 \mu M (compared with a mean value of 0.0089 \mu M for DEHP monoester) (38, 39). In our assay the mean concentration of the monoester metabolite of DEP found in human urine is in the concentration range necessary to for DEP to cause chemotaxis. Furthermore, our data suggest that H. pylori are an environmental source of DEP. In addition other environmental sources of DEP may be exposure to the volatile components of beauty care products such as perfumes, nail polishes, and hair sprays (39).

Despite the possibility that the production of DEP is strain-specific, it is the first description of a bacteria-produced small molecule that causes monocyte migration. As a small hydrophobic molecule, DEP may be able to rapidly diffuse through the mucus layer and the gastric epithelium to activate polymorphonuclear cells. DEP produced on the luminal side of the epithelium by non-invasive H. pylori may diffuse into the lamina propria, increasing local tissue concentration with a concomitant chemotactic effect on scattered resident monocytes. This chemotactic effect would contribute to the initiation of an inflammatory response further prompting monocyte migration to the site of inflammation. The DEP effect on neutrophils may be similar but was not directly tested (data not shown). In addition, DEP may act indirectly at the gastric epithelium and/or immune cells to illicit secretion of other chemotactic factors such as IL-8 (18) and monocyte chemotactic protein-1 (40, 41).

Chemotaxis plays a pivotal, inciting role in the inflammatory cascade and is an indicator of immune cell activation. The production of DEP by H. pylori provides a mechanism for the recruitment of inflammatory cells to the subjacent epithelium by a bacterial factor that may cross the epithelial boundary. The chemotaxis component of the inflammatory response has been demonstrated here for monocytes.

Comparison of the in vivo and in vitro effects of DEP to the heavily studied compound DEHP, a plasticizer widely used in polivinylchloride, demonstrates that, despite their similar structures (Fig. 8), DEP and DEHP do not have the same in vivo and in vitro effects. For example, DEHP has reproductive toxicity in animals (42, 43) while DEP does not (44). Furthermore, DEHP has been shown to be a peroxisome proliferator while DEP is not (45). Finally, DEP causes monocyte chemotaxis and DEHP does not (this work). The hazard potential of DEHP and its major metabolites to man has been extensively investigated (45–50) and these studies and other work confirm that DEP is not a known metabolite of DEHP in rats or in humans (51, 52).

Regardless of the source of DEP, this work demonstrates that it represents a new class of immune-modulatory agent; the full significance and implications for human disease remain to be defined. This work also provides a framework to understand how a purportedly non-invasive bacterium could stimulate a local inflammatory response that has known clinical consequences and may foster an environment favorable for H. pylori persistence. Knowledge of the structure of the chemotactic factor will allow the study of its role in the inflammatory process associated with H. pylori infections. It will be important to determine whether DEP causes release of inflammatory mediators from epithelial, mucosal, or immune cells, if its actions are receptor mediated, and what second messengers are activated by DEP in monocytes as well as other resident immune cells in the gastric mucosa.

Acknowledgments — Support from the CURE Peptide Biochemistry and Molecular Probes Core is gratefully acknowledged. In addition, the work of UCLA Student Research Project participant Babak Shabatian with some of the GC/MS experiments is gratefully acknowledged.

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Diethyl Phthalate, a Chemotactic Factor Secreted by *Helicobacter pylori*

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*J. Biol. Chem.* 2001, 276:48847-48853.

doi: 10.1074/jbc.M109811200 originally published online October 24, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M109811200

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