Role for Endotoxin in the Leukocyte Infiltration Accompanying Escherichia coli Inflammation

ANDREW C. ISSEKUTZ†* AND SHABIR BHIMJI

Departments of Microbiology and Pediatrics, Dalhousie University, Halifax, Nova Scotia, B3H 4H7 Canada

Received 12 August 1981/Accepted 23 December 1981

Escherichia coli organisms induce polymorphonuclear leukocyte (PMNL) infiltration during clinical infection and also in a rabbit dermal model of inflammation. We investigated the factors which may mediate this host response to E. coli. In vitro incubation of Formalin-killed E. coli in heat-inactivated rabbit plasma or balanced salt solution generated in the supernatant factors which induced in vivo PMNL infiltration upon intradermal injection into rabbits. However, these supernatants, in the presence or absence of plasma, did not induce PMNL migration in vitro. The in vivo activity was stable at 100°C and of high molecular weight (30,000). Antiserum to O antigen or to core glycolipid, but not to K or H antigen, as well as polymyxin B inhibited the release or activity of these E. coli-derived factors. The intradermal injection of 0.02 to 0.2 μg of four different endotoxin preparations or lipid A also induced marked PMNL infiltration in vivo. However, these preparations did not stimulate PMNL migration in vitro and failed to generate chemotactic activity in plasma except at very high concentrations (500 μg/ml). Anti-O serum inhibited PMNL infiltration induced by endotoxins with the corresponding O antigen and anti-core glycolipid serum inhibited all four endotoxins tested, whereas polymyxin B inhibited the activity of the endotoxins as well as that of lipid A. Base hydrolysis of endotoxin abolished PMNL infiltration. It is concluded that (i) endotoxin shed from E. coli (killed or live) may be one factor mediating the PMNL infiltration induced by this organism, (ii) endotoxin probably acts independent of in vivo complement activation, (iii) the activity is dependent on the lipid A moiety, and (iv) antibody binding to O or core glycolipid antigens can modify endotoxin so as to diminish its capacity to induce PMNL infiltration in vivo.

Escherichia coli and many other bacteria elicit a severe inflammatory reaction upon invasion of the tissues. We have studied the reaction produced by killed E. coli in a rabbit model suitable for quantitating various parameters of inflammation (17). In the accompanying paper (12), we report that immune serum or polymyxin B treatment of killed E. coli significantly diminished the magnitude and severity of inflammation induced by these bacteria when they were injected intradermally into rabbits. Such treatment diminished the vascular permeability or protein exudation, hyperemia, leukocyte infiltration, and vascular injury which normally are part of this inflammatory reaction (17).

Previous work on E. coli and other acute inflammatory reactions have suggested that the rate and degree of polymorphonuclear leukocyte (PMNL) infiltration of tissues is one factor determining the vascular permeability and hyperemic responses as well as the extent of vascular injury and hemorrhage (3, 14). In fact, the hemorrhage accompanying E. coli inflammation does not develop in rabbits made neutropenic with nitrogen mustard treatment (H. Z. Movat, M. Kopaniak, A. C. Issekutz, and B. J. Jeynes, Proc. 4th Int. Congr. Immunol., abstr. 15.8.10, 1980; M. Kopaniak and H. Z. Movat, manuscript in preparation; unpublished data). Because of the central role played by PMNLs in these reactions, we investigated some of the factors which may initiate PMNL infiltration in vivo during E. coli-induced inflammation and further studied the modulating effects of immune serum and polymyxin B.

MATERIALS AND METHODS

Measurement of leukocyte infiltration. Female New Zealand white rabbits (2.5 to 3 kg) had the hair on their back clipped, and 36 to 40 skin sites were designated. Leukocyte infiltration in response to various test injections given into these sites was quantitated with 51Cr-labeled leukocytes as described in detail previously (13) and in the accompanying report (12). Leukocyte infiltration was measured in a cumulative fashion, by

† Present address: Izaak Walton Killam Hospital, Halifax, Nova Scotia, Canada B3J 3G9.
injecting the test substances intradermally (0.2 ml) into the skin sites at the same time that the labeled leukocytes were injected intravenously. Leukocyte infiltration was allowed to proceed for 4.5 h, a time interval by which this process is 90% complete (13).

Measurement of PMNL chemotaxis in vitro. Chemotaxis of rabbit PMNLs was measured in vitro under agarose, using a modification of the technique described by Cutler (5) and Nelson et al. (23). The agarose consisted of 0.75% agarose type 2 (Sigma Chemical Co., St. Louis, Mo.) made up to contain isotonic minimal essential medium (MEM; Microbiological Associates, Walkersville, Md.), 0.5% crystaline bovine serum albumin (Sigma Chemical Co.), and 10 mM HEPES (N-2-hydroxyethyl) piperazine-N’-2-ethanesulfonic acid) buffer, pH 7.2 (Sigma Chemical Co.). A 7-ml portion of this solution (at 40°C) was poured into Falcon 3002 tissue culture dishes (60 by 15 mm; BBL Microbiology Systems, Cockeysville, Md.) and allowed to harden. A 10-ml amount of rabbit blood was collected into acid-citrate-dextrose anticoagulant, and 3 ml was collected into 30 U of heparin. From the latter, heparinized plasma was separated by centrifugation (500 × g for 15 min at 4°C). Leukocytes were prepared from the acid-citrate-dextrose blood by addition of 1 volume of 1% hydroxyethylcellulose (Polysciences Inc., Warrington, Pa.) to 4 volumes of blood to sediment the erythrocytes. The leukocyte-rich plasma was collected, and the leukocytes were harvested by centrifugation (200 × g for 10 min) and washed twice with Ca2+-Mg2+-free Tyrode solution. They were then resuspended to 3 × 105 PMNLs per ml in MEM containing 0.5% bovine albumin buffered with 10 mM HEPES (pH 7.2).

A positive chemotactic stimulus was generated by activating heparinized plasma with boiled, washed zymosan A particles (Sigma Chemical Co.). Two volumes of zymosan solution (15 mg/ml) or other agent or pyrogen-free saline (control) being tested for chemotactic factor generation were mixed with 1 volume of heparinized normal rabbit plasma (NRP) followed by 60 min of incubation at 37°C. Samples of these plasma mixtures were removed to test for residual hemolytic complement activity against sensitized sheep erythrocytes, using the technique of Kabat and Mayer (16). Remaining samples were incubated at 56°C for 30 min to inactivate residual complement before assaying for chemotactic activity. Bacteria-derived chemotactic stimuli were generated as described previously (33) by growing E. coli in MEM overnight. The MEM growth medium was then sterilized by centrifugation and membrane filtration (0.45 μm; Millipore Worthington, Malton, Ont.).

The chemotaxis assay was performed by punching in the agarose a series of three 2.4-mm-diameter wells situated 2.4 mm apart (23). The center well received 10 μl of leukocyte suspension, an adjacent well received 10 μl of the chemotactic stimulus, and the opposite well received 10 μl of control substance (MEM or saline-treated NRP, subsequently heat inactivated). The tissue culture dishes were incubated at 37°C in a humidified atmosphere for 2 h. Chemokinesis was measured by addition of 1 volume of test agent or control to 9 volumes of leukocyte suspension, adding this to a single well in the agarose and incubating for 3 h. The cells were fixed to the dishes by addition of methanol (10 min) and then 40% Formalin (10 min), after which the agarose was removed. The dishes were rinsed and stained with Wright stain, and cell migration was quantitated by using 30× magnification and an ocular micrometer. The furthest distance migrated by a uniform column of cells (which were 97% PMNLs) towards either the chemotactic stimulus-containing well or the control well was recorded. The results are expressed as the chemotactic difference, which is the migration in millimeters towards the chemotactic stimulus minus migration toward the control stimulus. Chemokinetic activity was measured by assessing the effect of the test agent on random migration.

Bacteria and antisera. The following E. coli serotypes were used to raise antisera in rabbits: O1:K1:H7, O18:H7:K1:H7, O7:K1, O55:K59, O127:B8, and the UDP-galactose epimerase-deficient E. coli mutant JS (kind gift from Peter Elsbach and Lorens Leive). The handling of the E. coli and the antiserum production were performed as described in the accompanying report (12).

Antisera were assayed for antibody by agglutination of Formalin-killed (O, K, H antigens) or boiled (O antigens) E. coli (25) in U-bottom microtiter plates as described in the accompanying report (12). Antibody to core glycolipid was quantitated with a modified enzyme-linked immunosassay described previously by Polin and Kennett (27) and in detail in the accompanying report (12). The titer of anti-J5 serum (anti-core glycolipid) against three different boiled E. coli serotypes was in the range of 1:10,000 to 1:30,000, whereas nonimmune (prebleed) titers were less than 1:40.

Endotoxin preparations. Four preparations of gram-negative endotoxins were studied, and their structure is shown schematically in Table 1. E. coli O55:K59 (BS) and O127:BS phenol-water-extracted endotoxins (Sigma Chemical Co.) contained <2% protein by the Polin method. Highly purified phenol-water-extracted endotoxin prepared from E. coli MRE600, a rough strain which does not synthesize a complete O antigen, was a kind gift from R. G. Johnson (National Research Council, Ottawa, Ont.). This endotoxin contained 1% protein. The criteria of purity for this endotoxin were as outlined by Johnson and Perry (15); i.e., the endotoxin eluted as a single symmetrical peak at the void volume on Sepharose 4B chromatography, contained no detectable ribose or deoxyribose, had no absorption maxima in the 210- to 300-nm UV region, and had a single absorption maximum at 460 nm when reacted with the carbocyanine dye assay for endotox- in. Salmonella minnesota R595 endotoxin extracted with phenol-chloroform-petroleum ether and lipid A obtained by mild acid hydrolysis of S. minnesota R595 endotoxin were purchased from List Biological Laboratories (Campbell, Calif.). This endotoxin contained 1% protein, <0.5% nucleic acid, 16% 2-keto-3-deoxyoctulosonate, and 7.6% phosphate according to the manufacturer. A lipid A preparation contained <0.16% 2-keto-3-deoxyoctulosonate. The O55, O127, and MRE600 endotoxins were dissolved in pyrogen-free water (Baxter Travenol, Malton, Ont.), as 1-mg/ml stock solutions, whereas R595 endotoxin was dissolved at 500 μg/ml in water containing 10 mM EDTA (as suggested by David Morrison). All stock solutions were stored in aliquots at −20°C, sonicated before use, and diluted in pyrogen-free saline (Baxter Travenol). Lipid A was solubilized in dimethyl sulfoxide as a
TABLE 1. Schematic representation of endotoxins used in these experiments

<table>
<thead>
<tr>
<th>Endotoxin</th>
<th>Endotoxin structure*</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli O55 or O127</td>
<td>Lipid A-KDO-core poly-O poly, Lipid A-KDO-core poly-O inactive</td>
</tr>
<tr>
<td>E. coli MRE600</td>
<td>Lipid A-KDO-core poly</td>
</tr>
<tr>
<td>E. coli JS</td>
<td>Lipid A-KDO-core poly</td>
</tr>
<tr>
<td>S. minnesota R595</td>
<td>Lipid A-KDO</td>
</tr>
<tr>
<td>Lipid A</td>
<td>Lipid A</td>
</tr>
</tbody>
</table>

* Based on references 11, 18, and 22. KDO, 2-Keto-3-deoxyoctulosonate; poly, polysaccharide.

stock solution of 5 mg/ml. This was then diluted in pyrogen-free saline so that the concentration of dimethylsulfoxide did not exceed 0.1%. This amount of dimethylsulfoxide did not influence the parameters tested. Base hydrolysis of endotoxin was carried out at 56°C for 60 min with 1 N NaOH as previously described (31).

Quantitation of endotoxin was performed with the Limulus amoebocyte lysate assay (Pyrotell; Cape Cod Associates), using the gel clot method as recommended by the manufacturer. Ultrafiltration of solutions was performed with Amicon ultrafiltration cells (10 ml) and membranes (Amicon Corp., Lexington, Mass.). Care was taken to use pyrogen-free solutions and plastic ware in all procedures.

RESULTS

In vivo leukocyte infiltration induced by E. coli.
To determine some of the host and bacterial factors which may initiate leukocyte infiltration in vivo in response to killed E. coli, we investigated the in vitro generation by E. coli of factors capable of inducing leukocyte infiltration. The capacity of killed E. coli to generate such activity in NRP was studied by incubating 10^8 killed E. coli (O1856;K1:H7) in 1 ml of 20% NRP and Hanks balanced salt solution (HBSS) for 60 min at 37°C. This concentration of NRP was found previously to generate sufficient complement-derived C5a (7, 13, 31) upon optimal activation by zymosan to induce an 80% maximal leukocyte infiltration response in vivo (13). After incubation, the bacteria were removed by high-speed centrifugation (4,000 × g for 20 min at 4°C), and the bacteria-free supernatants were heat inactivated (56°C for 30 min) and tested for induction of leukocyte infiltration upon intradermal injection into rabbits. The left-hand bar in Fig. 1 shows that injection of 0.2 ml of a suspension of 10^8 killed E. coli per ml in HBSS (2 × 10^7 per site), a dose approximating an 80% maximal response for this stimulus, induced a marked accumulation of 51Cr-labeled leukocytes (5,850 ± 425 cpm [standard error]). Control injection of HBSS or 20% NRP caused only 120 ± 18 cpm to accumulate. When killed E. coli bacteria at the same concentration were incubated in 20% NRP, activity inducing leukocyte infiltration was generated in the NRP-HBSS supernatant, which was nearly as great as that induced by the injection of the E. coli directly (4,250 ± 310 cpm). Heat inactivation of the plasma before incubation, to prevent the generation of complement-derived C5a chemotactic factor (7, 32), diminished only moderately the generation of activity in the supernatants (3,210 ± 280 cpm).

Furthermore, essentially the same amount of activity was generated or released into the supernatant upon incubation of the killed E. coli for 60 min in HBSS (3,005 ± 210 cpm). Histological sections of these lesions confirmed that >90% of the infiltrating leukocytes were PMNLs.

As a positive control in these experiments, the 20% NRP in HBSS was incubated similarly with zymosan (5 mg/ml) to activate complement and generate the C5a chemotactic factor, which is active in vivo (7, 13). The injection of these zymosan-treated supernatants induced marked leukocyte infiltration (2,850 ± 205 cpm), as described previously (13). However, in marked

FIG. 1. Induction of leukocyte infiltration by E. coli supernatants. The bar at far left represents the leukocyte response to killed E. coli O1856;K1:H7 injected intradermally (0.2 ml) in HBSS (10^7/ml or 2 × 10^7.02 ml). The remaining bars show the response to injection (0.2 ml) of bacteria-free supernatants. These were prepared by incubating E. coli (10^7/ml) or zymosan (2YMO; 5 mg/ml) for 60 min at 37°C in 20% NRP, heat-inactivated (56°C for 30 min) rabbit plasma (HIRP), or HBSS alone. The bacteria or zymosan was then removed by centrifugation (4,000 × g, 20 min), and the supernatant was heat inactivated (56°C for 30 min) before intradermal injection. The effect of injecting NRP, heat-inactivated rabbit plasma, or HBSS is also shown. Leukocyte infiltration was measured with 51Cr-labeled leukocytes. One representative experiment of three is shown. Values are means ± standard errors of quadruplicate measurements.
contrast to the situation with the killed E. coli, heat inactivation of the rabbit plasma before zymosan treatment completely prevented the generation of this activity (150 ± 25 cpm).

**In vitro chemotactic activity of E. coli supernatants.** The results in Fig. 1 suggested that during incubation of killed E. coli with NRP, heat-inactivated rabbit plasma, or HBSS a significant amount of complement-independent chemotactic activity for PMNLs in vivo was generated. This raised the possibility that HBSS-E. coli supernatants either contained factors directly chemotactic for PMNLs or generated chemotactic factors after incubation, for example, by activating complement in the rabbit with the generation of C5a in vivo. To evaluate these possibilities, the PMNL chemotactic activity of various supernatants was tested in vitro, using the chemotaxis-under-agarose technique. Table 2 shows the generation of chemotactic activity for rabbit PMNLs and the consumption of 50% hemolytic complement (CH50) when NRP was incubated with various activators. Zymosan treatment of NRP generated marked in vitro chemotactic activity (1.4 to 1.6 mm), which was easily detectable when this zymosan-activated plasma was diluted to even 1% (vol/vol) in MEM (1.0 ± 0.2 mm). Treatment with zymosan was associated with essentially complete consumption of CH50 in the plasma. Similarly, treatment of NRP with killed E. coli (O18ac:K1:H7) generated chemotactic activity (1.4 mm) and consumed CH50. However, the supernatant from killed E. coli incubated in HBSS (E. coli-HBSS supernatant), which induced marked leukocyte infiltration in vivo (Fig. 1), had essentially no chemotactic or chemokinetic (not shown) activity in vitro. In addition, incubation of the killed E. coli-HBSS supernatant with NRP (30%) did not generate appreciable chemotactic activity in the plasma and did not consume CH50 (<10%).

*E. coli* bacteria, during logarithmic growth phase, are known to produce chemotactic factors for PMNLs (29, 30, 33, 34). Therefore, a sterile culture filtrate of *E. coli* O18ac-K1:H7 grown in MEM was tested. This culture filtrate contained a significant amount of chemotactic activity for rabbit PMNLs (last line, Table 2).

**Effect of immune serum on generation of leukocyte infiltration-inducing activity.** To further investigate the nature of the activity released or generated by killed *E. coli* causing leukocyte infiltration, we made use of the observation in the accompanying report (12) that immune serum treatment of killed or live *E. coli* diminished the leukocyte infiltration they evoked. Figure 2 shows a representative experiment, similar to that shown in Fig. 1, with the exception that the *E. coli* (O18ac-K1:H7) were preincubated with either 0.3% nonimmune serum or 0.3% (3 agglutinating units) immune serum (anti-O18ac-K1:H7) for 60 min at room temperature following by washing. The injection into skin sites of nonimmune serum-treated *E. coli* (10⁹/ml; 2 × 10⁷ per site) induced marked leukocyte infiltration (5,110 ± 405 cpm). The activities of bacteria-free supernatants prepared as in Fig. 1 are also shown in Fig. 2. *E. coli* treated with immune serum generated significantly less activity in the supernatant (1,910 ± 250 cpm) when incubated with 20% NRP than nonimmune serum-treated *E. coli* (3,610 ± 275 cpm). This inhibitory effect of immune serum treatment was even more evident, expressed as percent inhibition, when immune serum-treated *E. coli* were incubated with heat-inactivated rabbit serum or in HBSS alone (inhibition, 65%).

The nature of the *E. coli* antigen and antibody reaction interfering with the *E. coli* release or generation in HBSS of factors inducing leukocyte infiltration was studied by using several antisera and two *E. coli* strains sharing the same K and H antigens but differing at the O antigen. Table 3 shows that antisera reacting at the K or H antigen or both did not significantly inhibit the generation of leukocyte infiltration-inducing activity in the supernatants (2 to 10%), whereas

<table>
<thead>
<tr>
<th>Attractant</th>
<th>Chemotactic activity (mm)</th>
<th>CH50 consumed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zymosan + NRP (30%) supernatant (ZAP)</td>
<td>1.40 ± 0.15</td>
<td>&gt;90</td>
</tr>
<tr>
<td>10% ZAP</td>
<td>1.60 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>1% ZAP</td>
<td>1.00 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>Killed E. coli + NRP (30%) supernatant</td>
<td>1.30 ± 0.12</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Killed E. coli-HBSS supernatant</td>
<td>0.04 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Killed E. coli-HBSS supernatant + NRP (30%)</td>
<td>0.07 ± 0.04</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Live E. coli culture filtrate (MEM)</td>
<td>1.03 ± 0.12</td>
<td></td>
</tr>
</tbody>
</table>

* a Substances were either tested directly by addition to the attractant well or incubated (60 min, 37°C) in 30% NRP to generate plasma-derived chemotactic activity. The plasma reactions were terminated by incubation at 56°C for 30 min before testing. Rabbit PMNLs were used as indicator cells. ZAP, Zymosan-activated plasma.

b Expressed as chemotactic difference or net migration towards attractant well. Random migration, i.e., towards control, ranged from 0.05 to 0.1 mm. Values are means ± standard errors of four experiments run in triplicate.

c Values are percent hemolytic complement (CH50) consumed in NRP by incubation (37°C for 60 min) with the test substance.
FIG. 2. Effect of immune serum on E. coli (E.C.) release of leukocyte infiltration-inducing activity. The bar at far left represents leukocyte infiltration induced by intradermal injection (0.2 ml; 2 x 10^7) of killed E. coli treated with 0.3% nonimmune serum (NIS) for 60 min at room temperature followed by washing. The remaining bars show the response to injection of bacteria-free supernatants prepared as in the legend to Fig. 1 except that the E. coli (10^8/ml) were pretreated as above with either nonimmune serum (0.3%) or immune serum (IS) (0.3%; 3 agglutinating units). These pretreated E. coli were then incubated (60 min, 37°C) in 20% NRP, 20% heat-inactivated rabbit plasma, or HBSS as in the legend to Fig. 1 to generate the active supernatants. One representative experiment of three is shown. Values are means ± standard errors of quadruplicate measurements.

antiserum reacting with O or a related antigen did inhibit (52 to 59%). These results were similar to those in the accompanying report (12) in which anti-O serum-treated E. coli bacteria were injected directly and were found to cause less leukocyte infiltration than nonimmune serum-treated E. coli.

Because these observations suggested that antibody binding to the O antigen of endotoxin played a role in inhibiting the release or activity of the factor(s) causing leukocyte infiltration, the effect of modifying the two other regions of the endotoxin molecule was investigated. Anti-serum to E. coli J5 (Table 3), which contains antibodies to the core glycolipid region (2), was found to inhibit (41 to 57%) when either O1:K1:H7 or O18wK1:H7 strains were used. Similarly, polymyxin B, which binds to the lipid A region of endotoxin (19), inhibited the release or activity by 47 to 59% when either strain of E. coli was tested. Finally, it was found that the in vivo activity of bacteria-free supernatants could be inhibited by 47 to 67% upon the addition of 1 to 3% of specific anti-O or anti-J5 serum or 5 μg of polymyxin B per ml to the active supernatant.

Induction of leukocyte infiltration by endotoxin in vivo. These observations suggested that the activity in the killed E. coli HBSS supernatants inducing leukocyte infiltration was related to endotoxin. This was supported by the detection of 1 to 10 μg of endotoxin per ml (by Limulus amoebocyte lysate assay) in supernatants prepared from killed or live E. coli, that the activity was heat stable (85% after 2.5 h at 100°C), and that >90% of the activity was retained on ultrafiltration by membranes with a cut-off of 30,000 daltons. To further investigate this possibility, we studied various endotoxin preparations for their effect on leukocyte infiltration in vivo. Endotoxins from different sources and ranging in structure from lipid A to the complete lipid A-core polysaccharide-O polysaccharide sequence were tested (see Table 1). Dose-response experiments indicated that the injection of as little as 0.0001 to 0.001 μg (depending on the preparation) of endotoxin induced easily measurable leukocyte infiltration, which histologically consisted mainly of PMNLs (>90%) during the first 4.5 h. The doses chosen for routine testing were selected to elicit an 80% maximal response, which approximated the maximal leukocyte response to 0.2 ml of undiluted zymosan-activated plasma. The doses ranged from 0.02 to 0.2 μg, and these doses induced a mean leukocyte infiltration of 3,110 to 4,825 cpm depending on the endotoxin preparations (Table 4).

<table>
<thead>
<tr>
<th>HBSS supernatant from E. coli</th>
<th>Inhibition (%) of leukocyte infiltration by treatment of killed E. coli with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-O1:K1:H7</td>
</tr>
<tr>
<td>O1:K1:H7</td>
<td>59 ± 5</td>
</tr>
<tr>
<td>O18wK1:H7</td>
<td>10 ± 5</td>
</tr>
</tbody>
</table>

* Formalin-killed E. coli (10^8/ml of HBSS) were incubated (60 min at room temperature) in polymyxin B (50 μg/ml), in 0.3 to 3% (i.e., 3 agglutinating units) of the indicated antiserum or 0.3 to 3% of the corresponding nonimmune serum. After being washed, these bacteria were incubated (60 min at 37°C) at 10^8/ml in HBSS, and the bacteria-free supernatants were tested for the induction of leukocyte infiltration by intradermal injection (0.2 ml) into rabbits.

* Results are percent inhibition of the activity in the E. coli supernatants due to antiserum or polymyxin B treatment of E. coli, relative to nonimmune serum or saline treatment of the E. coli. Values are means ± standard errors of at least three experiments performed in quadruplicate.
### TABLE 4. Effect of antiserum or polymyxin B on endotoxin-induced leukocyte infiltration

<table>
<thead>
<tr>
<th>Endotoxin (µg)</th>
<th>Inhibition (%) of leukocyte infiltration by addition of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-O55</td>
</tr>
<tr>
<td>E. coli O55 (0.1)</td>
<td>35 ± 3</td>
</tr>
<tr>
<td>E. coli O127 (0.2)</td>
<td>9 ± 9</td>
</tr>
<tr>
<td>E. coli MRE600 (0.02)</td>
<td>0 ± 4</td>
</tr>
<tr>
<td>S. minnesota R595 (0.1)</td>
<td>7 ± 5</td>
</tr>
<tr>
<td>Lipid A (0.5)</td>
<td>6 ± 6</td>
</tr>
</tbody>
</table>

*To various endotoxin preparations in saline was added either polymyxin B (1 µg per site) or the indicated antiserum (2%) or the corresponding nonimmune serum (2%). This mixture was then injected intradermally into rabbits, and the infiltration of leukocytes into the site was measured during the next 4.5 h.

*Results are expressed as percent inhibition of leukocyte infiltration due to addition of antiserum or polymyxin B relative to addition of nonimmune serum or saline. Values are means ± standard errors of at least four experiments performed in quadruplicate. The mean counts per minute in uninhibited endotoxin lesions were: O55 = 4,610; O127 = 4,505; MRE600 = 4,825; R595 = 3,305; and lipid A = 3,110; saline = 90 cpm.

In light of previous experiments suggesting that polymyxin B or antiserum to O polysaccharide or core glycolipid antigens of endotoxin could inhibit the leukocyte infiltration induced by killed *E. coli* (12) or supernatants prepared from these bacteria, we investigated the effect of such treatment on leukocyte infiltration induced by endotoxin injection. For this purpose, various antisera (2%) or polymyxin B (5 µg/ml; 1 µg per site) was added to the endotoxin solutions before injection. Polymyxin B was used in 10-fold or greater stoichiometric excess (20), whereas the antiserum concentrations were optimal based on preliminary dose-response experiments. Table 4 shows that addition of anti-O serum inhibited leukocyte infiltration induced by the corresponding O-antigen-bearing endotoxin (e.g., O55 and anti-O55), but not the response to a different O-bearing endotoxin or to *E. coli* MRE600 and *S. minnesota* R595 endotoxins, which lack a complete O-polysaccharide antigen. Addition of anti-J5 serum, which reacts primarily with the antigenically similar core polysaccharide and 2-keto-3-deoxyoctulosonate portion of the endotoxin molecule (2), inhibited the in vivo leukocyte response to four endotoxin preparations but not the response elicited by purified lipid A, which lacks 2-keto-3-deoxyoctulosonate or core polysaccharide. Finally, addition of polymyxin B inhibited the leukocyte response to all endotoxin preparations tested to varying degrees (33 to 69%) and almost completely abolished the leukocyte infiltration induced by lipid A (90%). Polymyxin B did not inhibit leukocyte infiltration to stimuli such as zymosan or zymosan-activated plasma (not shown).

Experiments were also performed with O55 endotoxin, in which the effect of combining anti-O55 or anti-J5 serum with polymyxin B was compared. Such combinations had a nearly additive effect in inhibiting leukocyte infiltration (anti-O55 = 35 ± 3%; inhibition; anti-J5 = 33 ± 3%; polymyxin B = 33 ± 2%; anti-O55 + polymyxin B = 52 ± 4%; anti-J5 + polymyxin B = 59 ± 3%). Mild alkali treatment of endotoxins is known to hydrolyze some of the fatty acids from lipid A with relative preservation of the 2-keto-3-deoxyoctulosonate and polysaccharide chain (reviewed in 17, 21). Therefore, to determine the role of the lipid A moiety in the observed effects, alkali hydrolysis of O55 and O127 endotoxins was performed. Such treatment decreased the leukocyte infiltration response by 95% (not shown).

**Induction of PMNL chemotaxis by endotoxin in vitro.** Because of the marked leukocyte infiltration induced by endotoxin in vivo, we determined in vitro whether endotoxin was chemotactic or chemokinetic for PMNLs and whether endotoxin may be exerting its in vivo effect through activation of humoral mediator systems, such as complement. Endotoxin concentrations ranging from 0.01 to 1000 µg/ml did not induce PMNL chemotaxis (Table 5) or chemokinesis (not shown) in vitro. To investigate the role of complement activation, 30% NRP was incubated for 60 min at 37°C with various concentrations of endotoxin at 10- to 50-fold increments. Addition of even 100 µg of O55 or MRE600 endotoxin per ml did not appreciably activate rabbit complement as judged by the generation of chemotactic activity or consumption of CHO.

Significant generation of chemotactic activity required the treatment of NRP with at least 500 µg of the two endotoxin preparations tested per ml.

**DISCUSSION**

Live or killed *E. coli* bacteria induce severe inflammatory reactions which are often accompanied by vascular damage (1, 17). Since the extent of PMNL infiltration is an important factor determining the degree of vascular injury and tissue damage in these and other acute inflammatory reactions (3, 14; Movat et al.,
TABLE 5. In vitro chemotactic activity of endotoxin

<table>
<thead>
<tr>
<th>Attractanta</th>
<th>Chemotactic activity (mm)b</th>
<th>CH50 consumed (%)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>O55 endotoxin (0.1–500 µg/ml)</td>
<td>0.03 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>MRE600 endotoxin (0.01–100 µg/ml)</td>
<td>0.02 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>O55 (0.5–100 µg/ml) + NRP (30%)</td>
<td>0.05 ± 0.03</td>
<td>&lt;10</td>
</tr>
<tr>
<td>O55 (500 µg/ml) + NRP (30%)</td>
<td>1.00 ± 0.15</td>
<td>33</td>
</tr>
<tr>
<td>MRE600 (0.05–100 µg/ml) + NRP (30%)</td>
<td>0.03 ± 0.02</td>
<td>&lt;10</td>
</tr>
<tr>
<td>MRE600 (1,000 µg/ml) + NRP (30%)</td>
<td>0.80 ± 0.10</td>
<td>25</td>
</tr>
</tbody>
</table>

a The endotoxins in saline were either tested directly by addition to the attractant well or incubated (60 min, 37°C) in 30% NRP to generate plasma-derived chemotactic activity. Plasma reactions were terminated by incubation at 56°C for 30 min before testing. Rabbit PMNLs were the indicator cells.

b Results are expressed as in footnote b, Table 2. Values are from four experiments.

c Expressed as in footnote c, Table 2.

Proc. 4th Int. Congr. Immunol., abstr. 15.8.10, 1980), we investigated the factors generated or released by E. coli which may mediate PMNL infiltration in vivo and PMNL migration or chemotaxis in vitro.

The C5a fragment generated upon complement activation of plasma or serum is one of the most potent PMNL chemotactic factors under in vivo or in vitro conditions (7, 13, 14, 32). Therefore, we investigated the generation in NRP by killed E. coli of factors inducing leukocyte infiltration in vivo and PMNL chemotaxis in vitro. Formalin-killed E. coli bacteria were studied because live E. coli bacteria are already known to produce chemotactic factors (29, 30, 33, 34). Figure 1 indicates that incubation of killed E. coli in NRP generated in the supernatant activity which in vivo caused marked leukocyte infiltration. However, this activity was only partly complement dependent as indicated by incubations with heat-inactivated rabbit plasma or HBSS. Under similar conditions, zymosan generated leukocyte infiltration-inducing activity only in complement-preserved plasma.

The in vitro results in Table 2, with the in vivo active E. coli supernatants, indicate that unlike plasma treated with zymosan (zymosan-activated plasma) or with killed E. coli, these supernatants were not chemotactic for PMNLs in vitro. Furthermore, these supernatants failed to generate C5a chemotactic activity or consume the hemolytic complement of plasma in vitro. This suggests that the in vivo activity of the supernatants is not due to in vivo complement activation by the supernatants. In this context, it should be pointed out that the in vitro chemotaxis assay was sensitive enough to detect the chemotactic activity present in as little as 1% zymosan-activated plasma. Thus, the activation of about 3% of the complement in 30% NRP should have been detectable. Table 2 also shows that sterile, bacteria-free culture filtrates from an overnight growth of live E. coli O18ac::K1:H7 contain a considerable amount of chemotactic activity for PMNLs. This is in agreement with previous reports of E. coli-derived peptides and lipids, which are chemotactic in vitro (29, 30, 33, 34). Taken together, the above results indicate that E. coli bacteria induce PMNL infiltration by at least three mechanisms: one involving complement activation by the E. coli with likely generation of C5a; a second mechanism involving the release of factors from the E. coli (demonstrable with killed organisms), which are not directly chemotactic or chemokinetic and exert their effect in vivo independent of complement; and a third mechanism involving the release from live E. coli of chemotactic peptides and lipids during their growth phase, as described previously (29, 30, 33, 34).

The nature of the factor(s) released by killed E. coli, which induce leukocyte infiltration in vivo, was investigated with a variety of antisera to E. coli as described in the accompanying report (12). Figure 2 shows that immune serum (anti-O18ac::K1:H7) treatment of killed E. coli O18ac::K1:H7 inhibited the release or activity of the factor(s). Table 3 shows that antibody to the O antigen of endotoxin or a related antigen, but not to K or H (K1:H7) or both, is required to inhibit the release or expression of the leukocyte infiltration-inducing activity. Furthermore, treatment of E. coli with antiserum to E. coli 15, which contains antibody to the immunologically cross-reacting core glycolipid region of endotoxin (2), or polymyxin B, which binds to lipid A (20), was also inhibitory. Finally, since the activity of the supernatants per se could be inhibited by addition of antiserum to the O antigen of the E. coli from which the supernatant was prepared, anti-J5 serum or polymyxin B (see above), it appears that endotoxin released from the E. coli during incubation was responsible for inducing leukocyte infiltration. This was further supported by the fact that the live or killed E. coli supernatants contained 1 to 10 µg of endotoxin per ml by the Limulus amoebocyte lysate assay and that the activity was stable (>85%) upon boiling for 2.5 h and was of high molecular weight (>30,000; 18, 22).

Subsequent experiments investigated the in vivo induction of leukocyte infiltration by various endotoxins. All preparations tested, both
complete and those deficient in O antigen or core polysaccharide (Table 1), were found to be potent inducers of in vivo leukocyte infiltration (Table 4). Table 4 shows that antibody binding to the O antigen of a specific endotoxin or to core glycolipid (anti-J5) inhibited the leukocyte infiltration induced by these endotoxins, while not altering the response to purified lipid A. The failure of antiserum to core glycolipid to alter the effect of lipid A is likely due to the poor antibody response to lipid A even when rough bacteria are used for immunization (9). We have so far been unsuccessful in raising high-titer antiserum to lipid A. Polymyxin B, which binds to lipid A and can neutralize many of the biological effects of endotoxin (4, 20, 22, 28), similarly inhibited the response elicited by various endotoxin preparations and almost completely abolished the response induced by lipid A. Lastly, alkali hydrolysis of O55 or O127 endotoxins, a process which cleaves fatty acids from lipid A (18, 22, 24), also nearly abolished (95%) the activity of these endotoxins. The latter observations indicate that the lipid A moiety is important for the induction of leukocyte infiltration by endotoxin. However, it appears that antibody to the specific O antigen of endotoxin or to the core glycolipid region may modulate lipid A effects, perhaps by steric hindrance or some other yet to be determined mechanism. The reason for incomplete inhibition by polymyxin B of the effect of endotoxin is not clear. It may, however, be due to incomplete binding of polymyxin B to endotoxin under certain conditions (19), resulting in only partial inhibition of lipid A effects, as observed previously by Niemets and Morrison (24).

In an attempt to determine the mechanism of induction of leukocyte infiltration by endotoxin in vivo, we investigated the effect of endotoxin on in vitro PMNL migration. Neither of the two endotoxin preparations tested induced PMNL chemotaxis (or chemokinesis [not shown]) in the in vitro assay (Table 5). Endotoxin at concentrations of 100 to 1,000 times higher than those inducing optimal leukocyte infiltration in vivo did not generate in NRP significant chemotactic activity for PMNL and did not significantly consume CH$_5O$ in the plasma. Endotoxin is known to activate complement via the classical or alternative pathway or via both (21, 22). However, the concentration of endotoxin required to generate chemotactic activity in NRP and to cause significant consumption of CH$_5O$ was 1,000- to 10,000-fold higher (500 to 1,000 µg/ml) than that required to induce leukocyte infiltration in vivo. These observations suggest that endotoxin, as was the case with the killed E. coli-HBSS supernatants, is not acting directly or through activation of complement to induce leukocyte infiltration. These findings help explain why complement depletion of rabbits with cobra venom factor diminished leukocyte infiltration only marginally in killed E. coli lesions (Movat et al., Proc. 4th Int. Congr. Immunol., abstr. 15.8.10, 1980; Kopaniak and Movat, in preparation; unpublished observations). The mechanism of action of endotoxin is unclear. Preliminary experiments, however, indicate that endotoxin in vivo may be inducing the generation of leukotrienes, some of which have potent chemotactic and chemokinetic activities (8, 10, 26).

In conclusion, our results indicate that nanogram quantities of endotoxin, released from E. coli at a site of infection, is probably one of several factors responsible for the leukocyte infiltration seen in these lesions. This action of endotoxin can be inhibited by antibody or polymyxin B binding to various regions of the endotoxin molecule. This suggests that polymyxin B or antiserum to “core” glycolipid may be a useful adjunct to bactericidal antibiotics in modulating leukocyte infiltration and thus the severity of inflammation associated with E. coli infections.

ACKNOWLEDGMENTS

We are grateful for the excellent technical help of J. Wilmshurst and the secretarial assistance of C. Maxham, K. Calhoun, and R. Sampson.

A.C.I. is supported in part by grant DG 209 and the work was funded by grant DG 211, both from the Medical Research Council of Canada.

LITERATURE CITED


