Neutrophil inhibition with L-selectin-directed MAb improves or worsens survival dependent on the route but not severity of infection in a rat sepsis model

Michael Haley, Chantal Parent, Xizhong Cui, Andre Kalil, Yvonne Fitz, Rosaly Correa-Araujo, Charles Natanson, Robert L. Danner, Steven M. Banks, and Peter Q. Eichacker

Critical Care Medicine Department, Clinical Center, National Institutes of Health, Bethesda, Maryland

Submitted 4 November 2004; accepted in final form 26 January 2005

ALTHOUGH EXCESSIVE HOST INFLAMMATION may play a role in the pathogenesis of sepsis (25, 30, 43, 47), anti-inflammatory agents designed to suppress this response have had limited success clinically for this lethal condition (17, 32). This may be because many host inflammatory mediators have divergent functions (6, 17, 31, 32). The neutrophil is an important cell’s migration into the extravascular space (8, 9, 23, 48). L-Selectin has been linked to the pathogenesis of several neutrophil-mediated inflammatory disorders, including sepsis, acute lung injury, ischemia-reperfusion injury, and renal allograft rejection (1, 2, 13, 26, 28, 29, 45, 46). Both HRL-3 and other L-selectin-directed monoclonal antibodies have been demonstrated previously to strongly influence neutrophil trafficking in the lung and other organs (24, 26, 28, 44). In the investigations reported here, the effects of HRL-3 were compared with control treatment in rats challenged with low, medium, or high doses of *Escherichia coli* (*E. coli*) bacteria via intravenous (IV) or intrabronchial (IB) routes.

MATERIAL AND METHODS

Animal care. The protocol used in this study was approved by the Animal Care and Use Committee of the Clinical Center of the

The potential influence of severity of infection on anti-inflammatory agents (17) was emphasized by a recent meta-analysis of published preclinical and clinical trials testing selective inhibitors of five different inflammatory mediators [tumor necrosis factor, interleukin-1, platelet activating factor receptor, bradykinin, and prostaglandin metabolites] showing that the effectiveness of these agents decreased as the severity of sepsis and its associated risk of death decreased (17). These findings were supported in prospective experiments in a rodent model of sepsis with selective inhibitors of tumor necrosis factor and superoxide anion (12, 17). Finally, on the basis of evidence of this relationship in a phase 3 trial, the Food and Drug Administration restricted the use of recombinant human activated protein C, an agent with anti-inflammatory as well as anti-thrombotic effects, to patients with severe sepsis (4, 18).

Route of infection may also alter anti-inflammatory agents (20, 35). Although route of infection in prospective preclinical studies did not alter the effects of tumor necrosis factor-directed agents, it did have a pronounced effect on granulocyte colony-stimulating factor (G-CSF), an agent designed to stimulate neutrophil function (33). In this case, however, G-CSF, although beneficial during extravascular infection, was sometimes harmful with intravascular infection.

The present study investigated the influence of severity and route of infection on the efficacy of leukocyte-selectin (L-selectin)-directed monoclonal antibody (MAb) HRL-3. L-Selectin (CD62L) is a constitutively expressed adhesion molecule present on neutrophils that participates in the initiation of this cell’s migration into the extravascular space (8, 9, 23, 48). L-Selectin has been linked to the pathogenesis of several neutrophil-mediated inflammatory disorders, including sepsis, acute lung injury, ischemia-reperfusion injury, and renal allograft rejection (1, 2, 13, 26, 28, 29, 45, 46). Both HRL-3 and other L-selectin-directed monoclonal antibodies have been demonstrated previously to strongly influence neutrophil trafficking in the lung and other organs (24, 26, 28, 44). In the investigations reported here, the effects of HRL-3 were compared with control treatment in rats challenged with low, medium, or high doses of *Escherichia coli* (*E. coli*) bacteria via intravenous (IV) or intrabronchial (IB) routes.

MATERIAL AND METHODS

Animal care. The protocol used in this study was approved by the Animal Care and Use Committee of the Clinical Center of the

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
National Institutes of Health. During the study, every effort was made to minimize animal suffering.

**Overall study design.** Male Sprague-Dawley rats (n = 565) weighing between 200 and 250 g and with central venous catheters placed 72 h before study were randomized to receive treatment with L-selectin MAb (HRL-3, 2 mg/kg sc, Burschinger Ingelheim Pharmaceuticals, Ridgefield, CT) or placebo (lyophilized hamster serum protein, 2 mg/kg sc, Sigma Chemical, St. Louis, MO) every 12 h for a total of three doses. Immediately before the second dose of HRL-3 or placebo, animals were again randomized to be challenged with one of two doses of *E. coli* either IV [0.75 or 2.0 × 10^9 colony-forming units (CFU)/kg] or IB (25 or 100 × 10^9 CFU/kg) designed to produce low or high mortality rates. Each bacterial dose was administered in 0.5 ml of phosphate-buffered saline (PBS). Animals receiving *E. coli* via one route were treated with PBS 0.5 ml alone via the other route as control. Once daily for 4 days beginning 4 h after inoculation, all animals received ceftriaxone (100 mg/kg im, Rocephin, Roche Laboratories, Nutley, NJ). Animals alive at 168 h postinoculation were considered survivors. Randomly selected animals at 6 h and survivors at 168 h were anesthetized and bled. Arterial blood gas analysis were performed before and lung lavage, histology, and wet-to-dry weight ratios after euthanasia. In additional experiments employing a similar protocol, animals (n = 235) were randomized to receive HRL-3, hamster serum protein placebo, or a nonspecific MAb placebo (HRL-2, 2 mg/kg sc, Boehringer Ingelheim Pharmaceuticals). Immediately before the second treatment, these animals were randomized to challenge with a medium dose of *E. coli* either IV (1.25 × 10^9 CFU/kg) or IB (60 × 10^9 CFU/kg). Animals received ceftriaxone and were observed as before. At 6 h in randomly selected animals or at 168 h in all survivors CBC, arterial blood gas analysis, quantitative blood bacteria counts, and lung lavage were performed.

**Study agents.** L-Selectin MAB (HRL-3) was prepared from Armenian hamster ascites (Dr. Robert Rothlein, Boehringer Ingelheim Pharmaceuticals). HRL-3 is a hamster MAb of the IgG class that specifically recognizes rat L-selectin adhesion protein (44). HRL-3 blocks L-selectin from binding to its complementary carbohydrate ligand on the endothelial cell and thus inhibits neutrophil rolling along the endothelium. HRL-2 is a nonspecific antibody (44).

**Bacterial inoculation.** In brief, *E. coli* 0111:B4 was stored in 1-ml aliquots of bactopeptone broth (Difco, Detroit, MI) and glycerol at −70°C. For preparation of bacteria, an aliquot of frozen culture was thawed and inoculated into 500 ml of brain-heart infusion broth. The concentration of bacteria in the final suspension was estimated by plating successive 10-fold dilutions of the bacterial suspensions onto MacConkey agar and later scoring of visible colonies after 24 h of incubation at 37°C. For inoculation, all animals were anesthetized with an intramuscular injection of ketamine (40 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (20 mg/kg; Miles, West Haven, CT). IV *E. coli* inoculation or PBS control was administered via a central venous catheter. For IB *E. coli* inoculation or PBS control, animals were placed in the dorsal recumbent position and intubated endotracheally under direct visualization by use of a 20-gauge plastic arterial catheter. Animals were recovered in a prone position on absorbent tissue. They were housed in plastic Plexiglas cages and observed every 2 h for the first 12 h and then two times daily.

**Blood and lung neutrophil, lung injury, and bacteria measurements.** Animals were anesthetized with a mixture of ketamine (40 mg/kg) and xylazine (20 mg/kg). Arterial blood for CBC and differential (ZB1, Coulter Electronics, Hialeah, FL) for neutrophil determination and blood-gas analysis (ABL-300, Copenhagen, Denmark) was obtained from the tail artery. Alveolar-to-arterial oxygen gradients were determined as previously described (15). With use of sterile technique, cardiac puncture was performed for quantitative blood cultures. These were collected in isolator tubes (1.5 ml, DuPont Medical Products, Wilmington, DE), and serial dilutions of lysed samples were plated for bacterial count. After blood sampling, the animals were euthanized via cervical dislocation and their lungs were removed. For wet-to-dry ratio determination, one lower lobe was removed, immediately weighed, air-dried for 1 wk, and then reweighed. Animals were then randomly selected for either lung lavage or histological tissue analysis.

Lung lavage was performed by cannulating the trachea with a metallic cannula and sequentially lavaging the lungs with four 3-ml aliquots of 0.9% saline. These aliquots were combined, and 1 ml was placed in a sterile tube for quantitative bacterial count. The remaining lavage solution was centrifuged, the supernatant removed, and the cell button resuspended. Cells counts were performed with an electronic cell counter (ZBI, Coulter Electronics). Slides for differential cell count were prepared (Cytospin 2, Shannon Instrument, Sewickley, PA) and stained (Hematek Slide Stainer, Baxter Scientific, Cockeysville, MD). Cell differential was then performed, and neutrophil concentrations were calculated. Lavage supernatants were passed through a 45-μm filter, and protein determinations were performed by use of the Folin-Lowry method (19).

For histological analysis, the left lower lobe bronchus was cannulated and the lobe was infused and fixed with formaldehyde (4%; Mallinkrodt Specialty Chemicals, Paris, KY), dehydrated, embedded in paraffin, and sectioned in 6-μm thicknesses. As previously described, lung tissue sections were analyzed in a blinded fashion, and the degree of injury was graded [0 (absent) to 5 (severe)] for six different parameters including perivascular edema, intra-alveolar edema, hemorrhage, fibrin, congestion, and hyperplasia or swelling of alveolar duct and bronchiolar epithelium (19). The number of alveolar neutrophils was determined by randomly examining 10 fields at high magnification (oil immersion, magnification ×480) (19).

**Statistical methods.** Survival data were analyzed with a Cox Proportional Hazards model for treatment (HRL-3) and *E. coli* challenges [IV (doses: 0.75, 1.25, or 2.0 × 10^9 CFU/kg) and IB (doses: 25, 60, or 100 × 10^9 CFU/kg)]. Additionally, because the Cox hazard model showed no significant interaction based on different doses of *E. coli*, administered within a given challenge group (IV or IB), data were pooled to give an overall effect of HRL-3 on mortality for each route of challenge. Thus eight separate survival analyses (three *E. coli* doses alone and combined for each route) were performed. Hazards ratios with accompanying standard errors are presented to facilitate data presentation. Intravascular neutrophils (CBC and histology) and intra-alveolar neutrophils (lung lavage and histology) were enumerated. ANOVA was performed for each representative group. Because each ANOVA indicated that no significant interaction based on the dose of *E. coli* was present within a given challenge group (IV or IB), the data were pooled to facilitate data presentation. The degree of lung injury was assessed by identifying nine separate physiological and histological parameters. An ANOVA was performed on each parameter of lung injury for each dose of *E. coli* in both challenge groups. Because each ANOVA indicated that no significant interaction based on the dose of *E. coli* was present within a given challenge group (IV or IB), the data were pooled to facilitate data presentation. Additionally, an overall lung injury score was calculated as previously described (19). Briefly, from each of the nine parameters, the mean score for the group of interest (HRL-3) was subtracted from the mean score of the control group. Thus a positive or negative score reflects a deviation of the treatment group (HRL-3) from that of the control group.

**RESULTS**

**Effect of HRL-3 on mortality rates.** Compared with the hamster serum protein control, with medium doses of *E. coli* administered via either IV or IB routes, the nonspecific antibody HRL-2 produced mortality rates that did not differ sig-
nificantly [11 of 30 serum protein animals vs. 15 of 31 HRL-2 animals died with IV *E. coli* and 4 of 28 serum protein animals vs. 6 of 30 HRL-2 animals died with IB *E. coli*, *P* = not significant (NS) for both routes]. Therefore, the survival data from these control groups were combined for each individual route. Similar for each route of infection and with both placebo and HRL-3, the highest dose of *E. coli* administered produced significantly greater mortality rates compared with lower doses (*P* ≤ 0.0001) (Fig. 1). Route but not dose of *E. coli* altered the effects HRL-3 on mortality rates (Figs. 1 and 2). Similarly (*P* = NS) with each dose of IV *E. coli* challenge, compared with control, HRL-3 reduced mortality rates (mean hazards ratio ± SE) both early (0–6 h; −0.75 ± 0.23, *P* = 0.001 for all *E. coli* doses combined) and late (6–168 h; −0.72 ± 0.36, *P* = 0.04 for all *E. coli* doses combined) (Fig. 2). In contrast, similarly (*P* = NS) with each dose of IB *E. coli*, compared with control, HRL-3 reduced the hazards ratio early (−1.1 ± 0.36, *P* = 0.002 for all *E. coli* doses combined) but worsened it late (0.87 ± 0.23, *P* = 0.002 for all *E. coli* doses combined). The differing effects of HRL-3 on mortality rates from early to late time periods comparing infection via IV and IB routes were highly significant (*P* < 0.0001) (Fig. 2).

**Effect of HRL-3 on blood and lung neutrophils.** Compared with the hamster serum protein control, with medium doses of *E. coli* administered via either IV or IB routes, the nonspecific antibody HRL-2 produced changes in blood and lung lavage neutrophils that did not differ significantly (*P* = NS). Therefore the data from these control groups were combined for analysis. Overall, with both routes of infections, at 6 h blood neutrophils both on CBC and vascular histology were reduced, compared with 168 h, and these reductions were greater with high compared with low doses of *E. coli* (*P* ≤ 0.01 for each comparison) (Tables 1 and 2). Neither route nor dose of *E. coli* infection altered the effects of HRL-3 on blood neutrophils. Early at 6 h and similarly (*P* = NS) with each dose and route of *E. coli*, compared with control, HRL-3 resulted in small but significant reductions in blood neutrophils (*P* ≤ 0.0004 for both routes of infection combined across *E. coli* dose) (Tables 1 and 2, Figs. 3 and 4A) except for neutrophils on vascular histology with IV *E. coli* that were reduced but not significantly (*P* = NS). Late at 168 h and similarly (*P* = NS) with each dose and route of *E. coli* challenge, compared with control, HRL-3 did not alter blood neutrophils significantly (*P* = NS for all) (Tables 1 and 2, Figs. 3 and 4A). Compared with 6 h, neutrophils on vascular histology at 168 h were significantly increased with HRL-3 (*P* = 0.001).

Overall, with both routes of infection at 6 h lung neutrophils both in lavage and on histology were increased, but, compared

---

**Fig. 1.** Proportion of animals surviving after challenge with 1 of 3 increasing doses of *Escherichia coli* via intravascular [IV; 0.75, 1.25 or 2.0 × 10^9 colony-forming units (CFU/kg)] or intrabronchial (IB; 25, 60 or 100 × 10^9 CFU/kg) routes and treatment with HRL-3 or control (i.e., hamster serum protein alone with low and high *E. coli* doses and together with nonspecific HRL-2 MAb with medium *E. coli* doses).

**Fig. 2.** Effect of HRL-3 treatment on the hazards ratio early (0 to 6 h) or late (6 to 168 h) after challenge with all doses of *E. coli* combined via IV or IB routes. With IV *E. coli*, HRL-3 was beneficial and reduced the hazards ratio both early and late. In contrast, with IB *E. coli*, although HRL-3 was beneficial and decreased the hazards ratio early, by 168 h it was harmful and increased the hazards ratio. Overall the differing effects of HRL-3 on the hazards ratio early and late after IB *E. coli* were highly significant. Furthermore, the differing effects of HRL-3 on hazards ratio early and late comparing infection via IV and IB routes were also highly significant (*P* < 0.0001). NS, not significant.
with 168 h, these increases were greater for IB compared with IV E. coli challenge (P ≤ 0.01 for each comparison) (Tables 1 and 2). Overall at 168 h, lung lavage neutrophils were greater with IB compared with IV E. coli (P = 0.001). Route but not dose of E. coli altered the effects of HRL-3 on lung neutrophils. With IV E. coli, compared with control, both early at 6 h and late at 168 h, HRL-3 did not significantly alter lung neutrophils either in lavage or on histology (P = NS for all combined across E. coli dose) (Tables 1 and 2, Figs. 4B and 5). In contrast, with IB E. coli, compared with control HRL-3 reduced lung neutrophils both in lavage and on histology early at 6 h (P ≤ 0.001 for each parameter combined across E. coli dose) but increased each measure late at 168 h although not significantly (P = NS for each). Compared with early decreases, however, later increases in both measures of lung neutrophils with HRL-3 after IB E. coli occurred in a pattern that was significantly different (P ≤ 0.007 for both combined across E. coli dose).

Although HRL-3 was associated with early reductions in both blood and lung neutrophils, these decreases appeared substantially greater in the lung after IB E. coli. A comparison showed that decreases in neutrophils at 6 h with HRL-3 were more than sixfold greater on alveolar compared with vascular histology, two measures that were similarly conducted (Fig. 4).

Effect of HRL-3 on lung injury. To determine the effects of HRL-3 on lung injury, animals receiving the lowest and highest doses of E. coli with each route of infection in which all nine lung injury parameters were determined were analyzed. Route but not dose of E. coli altered the effects of HRL-3 on lung injury and changes with low and high E. coli doses were combined (Table 3). With IV E. coli, compared with control both early at 6 h and late at 168 h, HRL-3 increased some lung injury parameters and decreased others, and the effect of treatment on the overall lung injury score was not significant at either time point (P = NS for both) (Table 3, Fig. 6). In contrast, with IB E. coli, HRL-3 decreased all nine parameters of lung injury early at 6 h and increased all nine late at 168 h (Table 3, Fig. 6). As a result, with IB E. coli, HRL-3 significantly reduced the overall lung injury score early (P = 0.0001) and significantly increased it later (P < 0.0008), in patterns that were very different between the two time points (P < 0.0001) (Fig. 6). Furthermore, the differing effects of HRL-3 on lung injury early and late comparing IV and IB E. coli was highly significant (P < 0.0001) (Fig. 6).

HRL-3 effects on bacterial cultures from blood, lung tissue, and lung lavage. Overall, with both routes of infection, compared with 168 h, at 6 h blood bacteria counts were increased and these increases were greater with high compared with low doses of E. coli challenge (P ≤ 0.05 for all comparisons, data not shown). Compared with 168 h, at 6 h lung bacteria in tissue and lavage were also increased, and these increases were greater with IB compared with IV E. coli challenge (P ≤ 0.05 for all comparisons). Compared with control, HRL-3 did not alter bacteria counts in either blood or lung at either early or late time points (P = NS for all).

Table 1. Mean neutrophil counts after placebo, low-dose, or high-dose Escherichia coli challenge

<table>
<thead>
<tr>
<th>E. coli challenge</th>
<th>6 h</th>
<th>168 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Route and Dose (CFU × 10^9/kg)</td>
<td>Treatment</td>
<td>Intravascular neutrophils</td>
</tr>
<tr>
<td></td>
<td>CBC, cells × 10^6/mm^3</td>
<td>Histology, cells/HPF</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>0.75 Control</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>0.75 HRL-3</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>2.0 Control</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>2.0 HRL-3</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>IB</td>
<td>25 Control</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>25 HRL-3</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>100 Control</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>100 HRL-3</td>
<td>0.1 ± 0.0</td>
</tr>
</tbody>
</table>

Values are means ± SE. CBC, complete blood count; Histology, cells/HPF; Lavage, cells/µl; IV, intravascular; IB, intrabronchial.

Table 2. Mean neutrophil counts, alveolar-arterial oxygen gradient, and lavage protein content after placebo or medium dose Escherichia coli challenge

<table>
<thead>
<tr>
<th>E. coli challenge</th>
<th>6 h</th>
<th>168 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Route and Dose (CFU × 10^9/kg)</td>
<td>Treatment</td>
<td>CBC neutrophils, cells × 10^6/mm^3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>1.25 Placebo</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>1.25 HRL-2*</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>1.25 HRL-3</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>IB</td>
<td>60 Placebo</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>60 HRL-2*</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>60 HRL-3</td>
<td>0.1 ± 0.0</td>
</tr>
</tbody>
</table>

Values are means ± SE. AaO2, alveolar-arterial oxygen gradient; HRL-2, nonspecific antibody control.
In this rat model of sepsis, route but not severity of infection had a substantial influence on the effects of L-selectin inhibition with HRL-3. With low and high doses of *E. coli* administered via the intravascular route, this treatment reduced mortality rates both early and late and did not impair lung function. In contrast, HRL-3 treatment with low and high doses of IB *E. coli*, despite an initial improvement in both mortality rates and lung injury, was ultimately associated with increased mortality rates and worsened lung injury. There are several potential explanations for these differing effects.

Activated neutrophils may have had a limited role in host defense with intravascular *E. coli* challenge. Evidence exists that reticuloendothelial cell clearance of bacteria independent of the neutrophil plays an important role in the clearance of...
Table 3. Effect of HRL-3 on 9 parameters of lung injury after Escherichia coli challenge

<table>
<thead>
<tr>
<th>Time, h</th>
<th>AaO₂, Torr</th>
<th>Lavage Protein, mg/dl</th>
<th>Lung W/D, %</th>
<th>Alveolar Edema</th>
<th>Alveolar Hemorrhage</th>
<th>Alveolar Fibrin</th>
<th>Congestion</th>
<th>Hyperplasia</th>
<th>Perivascula r Edema</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0.19±0.21</td>
<td>−0.12±0.08</td>
<td>0.18±0.05</td>
<td>−0.21±0.11</td>
<td>0.34±0.12</td>
<td>0.14±0.12</td>
<td>0.19±0.10</td>
<td>0.30±0.10</td>
<td>−0.57±0.11</td>
</tr>
<tr>
<td>168</td>
<td>0.02±0.08</td>
<td>−0.24±0.04</td>
<td>−0.06±0.08</td>
<td>−0.09±0.09</td>
<td>−0.30±0.10</td>
<td>−0.13±0.08</td>
<td>−0.38±0.11</td>
<td>0.30±0.12</td>
<td>−0.30±0.11</td>
</tr>
<tr>
<td>6</td>
<td>−0.03±0.09</td>
<td>−1.07±0.08</td>
<td>−0.03±0.06</td>
<td>−0.71±0.11</td>
<td>−0.58±0.12</td>
<td>−0.81±0.14</td>
<td>−0.24±0.09</td>
<td>−0.92±0.10</td>
<td>−0.96±0.10</td>
</tr>
<tr>
<td>168</td>
<td>0.44±0.06</td>
<td>0.30±0.07</td>
<td>0.15±0.17</td>
<td>0.39±0.08</td>
<td>0.11±0.09</td>
<td>0.09±0.07</td>
<td>0.10±0.10</td>
<td>0.47±0.11</td>
<td>0.31±0.10</td>
</tr>
</tbody>
</table>

Values are means ± SE. W/D, wet-to-dry weight ratio.

infection originating in the intravascular space (39, 41). However, intravascular *E. coli* challenge likely also resulted in endothelial cell activation (5, 38). Widespread adhesion of activated neutrophils to endothelium, in the absence of a localized nidus of infection, may have maximized the harmful and limited the beneficial effects of this interaction. Under these circumstances, inhibition of L-selectin with HRL-3 may have done little to alter essential host defense mechanisms but had substantial beneficial anti-inflammatory effects.

Early release of endotoxin or other bacterial products into the vascular space after IB challenge with *E. coli* may have also initially caused widespread activation of neutrophils and endothelial and inflammatory injury. This injury would have been most pronounced in the lung where the challenge was concentrated. The early beneficial effects of HRL-3 both on survival and lung injury are consistent with this. In contrast to IV *E. coli*, however, IB challenge ultimately resulted in an established nidus of infection where activated neutrophils would have had an important protective role in the innate immune response. Inhibition of pulmonary neutrophil recruitment by HRL-3 with IB *E. coli* as was manifested by early reductions in lung lavage and histology neutrophil counts may have resulted in more extensive infection and secondary injury. Later reductions in survival rates and worsened lung injury with HRL-3 at 168 h are consistent with this. Although neither blood nor lung bacteria counts were increased in animals receiving HRL-3, this may be because administration of antibiotics 2 h before laboratory measures in the present study altered the results of microbiology testing. It is also possible that the microbiology techniques employed were insensitive to the effects HRL-3 may have had on bacterial clearance. Consistent with a potentially adverse effect of HRL-3 on host defense in the present study, however, in a recently published phase 2 clinical trial in trauma patients, another L-selectin-directed MAbs was found to be associated with dose-related increases in the rate of infection (40). Alternatively, however, the detrimental effects of HRL-3 on survival rates or lung injury during IB infection in the present study may have been because it interfered either with a later adaptive immune response necessary for the clearance of an IB nidus of infection or with subsequent reparative processes.

The present findings with HRL-3 are consistent with prior studies performed in this laboratory in a large animal model, in which MAbs directed at leukocyte integrin adhesion molecules improved survival with IV tumor necrosis factor challenge but worsened it with intraperitoneal *E. coli* infection (14, 16). They are also consistent with studies in this rat model of pneumonia in which integrin MAbs, although reducing early lung neutrophil recruitment and injury, increased both at later time points and worsened survival (19). Finally, they are consistent with studies in which neutrophil stimulation with G-CSF, although harmful in several different models employing IV bacteria challenge, improved survival with extravascular bacterial challenge (33). Overall, our studies with adhesion molecule-directed MAbs and G-CSF indicate that the effects of modulating neutrophil function may be greatly influenced by the site of the infectious challenge. These studies suggest that the neutrophil’s role in these models in clearing an extravascular site of infection appears critical and is net beneficial. Thus, if bacterial infection is predominantly extravascular, neutrophil augmentation is likely beneficial, whereas its inhibition may be harm-
ful. In contrast, neutrophil activation has the potential to contribute to host injury during systemic inflammation associated with infection present primarily in the vascular space. In this case, neutrophil stimulation may be harmful but inhibition beneficial.

Stimulation of oxidant activity is central to the host inflammatory response during infection (37, 49). During this process, neutrophil and endothelial interactions via selectin or other adhesive mechanisms can both result from as well as contribute to the release of superoxide, nitric oxide, peroxynitrite, and other oxidant products (22, 36, 42, 50). In the present study, therefore, inhibition of L-selectin with HRL-3 had the potential to inhibit both oxidant-stimulated neutrophil and endothelial interactions as well as oxidant production resulting from such interactions.

In contrast to other studies in this rat model in which severity of infectious challenge significantly altered the effects of tumor necrosis factor and superoxide anion inhibition, changing the dose of E. coli challenge and its associated mortality rate did not influence HRL-3 with either route of infection (12, 17). This suggests that the relative influence of these two conditions on individual components in the inflammatory response as well as agents that target them may vary. The importance of the neutrophil for microbial clearance from the extravascular space may make any agent harmful that directly interferes in this response, regardless of the severity of the underlying infection and its associated inflammatory response. In combination, these findings suggest that preclinical studies assessing the effects of new anti-inflammatory agents for sepsis may have to investigate both of these conditions and potentially others.

Although lung injury and survival were both improved by HRL-3 after 6 h observation in animals challenged with IB E. coli, overall survival and lung injury were ultimately worsened by 168 h. Thus preclinical sepsis models evaluating new therapies for sepsis must account for the possibility that some agents, while potentially having short-term beneficial effects, may be detrimental with longer observation. The preclinical assessment of such therapies should include models that permit both short- and long-term study.

Although HRL-3 is recognized to inhibit lung neutrophil-endothelial interactions and lung neutrophil recruitment, in the present study it also caused reductions in circulating cells. These decreases may have been related to the use of a whole antibody as opposed to F(ab)_2 fragments only (28). Thus decreases in lung neutrophils with IB E. coli at 6 h with HRL-3 may have been related both to reduced blood neutrophils as well as inhibition of endothelial interactions. However, the latter mechanism still appears to have had an important role. Review of Figs. 3 to 5 shows that reductions in both the concentration of neutrophils in lung lavage and the number of neutrophils enumerated on alveolar histology were substantially greater compared with reductions on CBC and vascular neutrophils enumerated on alveolar histology. Furthermore, both in vitro and in vivo studies have demonstrated the importance of L-selectin in mediating extravascular neutrophil recruitment and the direct inhibitory effects that HRL-3 and other agents may have on this recruitment (1, 2, 8, 9, 13, 23, 26, 28, 29, 44–46, 48).

The model employed in the present study was developed and applied previously to reproduce many of the conditions encountered during clinical sepsis (12, 17, 19, 33, 34). IB administration of bacteria results in an intrapulmonary nidus of infections, which simulates pneumonia, a common cause of sepsis today. The dose of bacteria administered can be altered to simulate the carrying severities of infection that are encountered clinically. Also, antibiotics are employed just as they are clinically. However, as with any sepsis model, the present one has limitations. This model does not include ventilatory or vasopressor hemodynamic support, which are frequently required in patients with severe sepsis. Furthermore, rodents may be dependent on mechanisms of host defense different from those in the human. Finally, unlike an animal model, patients presenting with sepsis vary widely with respect to age, underlying medical conditions, and genetic predispositions. Differences such as these may provide a basis for anti-inflammatory agents like the one tested here to have differing effects comparing this model and humans.

In conclusion, the findings from the present study suggest that route of infection may have an important influence on anti-inflammatory agents designed to alter neutrophil recruitment during sepsis. Clinically, patients with sepsis, although a diverse group, typically present with sepsis related to an extravascular site of infection. These animal studies suggest that therapies that strongly inhibit neutrophil trafficking will have limited application in such patients. However, in a small group of patients in which the onset of sepsis may be associated with the sudden introduction of a high concentration of intravascular bacteria, such as during the irrigation of an infected indwelling catheter, treatment with such agents may have a role. Unfortunately, early in the course of sepsis, it may be very difficult to define important characteristics of the underlying infection, including its route, type, or severity. The present findings as well as others, however, emphasize how important this definition may be, especially if immunomodulatory therapies directed at host mediators with divergent effects like the neutrophil are to be applied effectively in patients (17). Thus, although developing therapies to modulate the inflammatory response during sepsis is important, it appears essential that research also be directed at identifying markers that will permit rapid and accurate characterization of the underlying causes of sepsis. Until such markers are identified, agents with the potential to inhibit the host’s inflammatory response during infection must be administered with great caution.

ACKNOWLEDGMENTS

The authors thank Lisa Ruprecht for preparation of the manuscript.

REFERENCES


