Cecal ligation and puncture induced sepsis impairs host defense against Enterococcus faecium peritonitis

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Abstract

Purpose: Multiresistant and vancomycin resistant Enterococcus faecium (VRE) can cause serious infections in hospitalized patients with various co-morbid diseases. We investigated the course of VRE peritonitis after cecal ligation and puncture (CLP)-induced sepsis and compared this to sham operated mice.

Methods: Mice were subjected to CLP or sham surgery. Forty-eight hours thereafter four groups were created by subjecting mice to peritoneal injection of either VRE or saline.

Results: Mice infected with VRE after CLP were severely impaired in eliminating VRE from the peritoneal cavity and distant body sites. These mice failed to mount an early inflammatory response at the primary site of VRE infection. VRE superinfection did not influence CLP-induced organ damage or polymicrobial bacterial loads.

Conclusions: Sublethal polymicrobial sepsis greatly facilitates infection and dissemination of VRE. VRE does not influence the course of CLP-induced sepsis.

Keywords

Enterococcus faecium · Cecal ligation and puncture (CLP) · Innate immunity · Immunoparalysis · Sepsis · Mouse model

Introduction

Infections with multiresistant and vancomycin resistant Enterococcus faecium (VRE), are a growing problem worldwide [1]. Severe infections with VRE are almost exclusively found in immunocompromised patients, including patients at intensive care units suffering from different co-morbid conditions [2–4].

Patients who have survived the initial phase of sepsis demonstrate features consistent with immune depression. It has been proposed that this immune suppression contributes to the enhanced susceptibility to nosocomial infections and late mortality of sepsis patients [5–11]. Cecal ligation and puncture (CLP) is considered a clinically relevant model to study the septic response [12]. Many studies that investigated the sepsis-induced state of immunoparalysis used the model of CLP, especially to examine host defense against secondary pneumonia [13–15]. VRE is often cultured from abdominal infections, frequently resulting in subsequent dissemination to
blood and distant organs [16, 17]. To investigate the immune response to VRE infection we previously set up a model of VRE peritonitis in mice [18]. Healthy mice are able to mount an effective immune response in this model with peak cytokine levels after 2–6 h and an early peritoneal neutrophil influx, resulting in clearance of the systemic infection in 2–3 days. We here aimed to obtain insight into the innate immune response to VRE infection in an immunocompromised host, seeking to mimic the clinical scenario of abdominal sepsis with superimposed VRE peritonitis. For this, mice were subjected to CLP or sham surgery and subsequently infected with VRE intraperitoneally.

Materials and methods

Mice

Specific pathogen-free 10-week-old female C57BL/6 mice were purchased from Harlan Sprague-Dawley (Horst, The Netherlands). The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

Cecal ligation and puncture

CLP was performed as previously described [19]. Mice were given buprenorphine (Temgesic®, Schering-Plough, Amstelveen, The Netherlands) 0.075 mg/kg subcutaneously 30 min preoperatively, and were anesthetized via inhalation of a mixture of O2 (1–2 l/min) and isoflurane 2.0–2.5% (Burtons, Kent, UK). During surgery mice were kept on a heating pad at 37°C (Animed, Barneveld, The Netherlands). A 1-cm midline incision was made on the abdomen, and the cecum was exposed. The distal 1 cm of the cecum was ligated with a 4–0 Vicryl suture (Ethicon, Johnson&Johnson, St-Stevens-Woluwe, Belgium) and punctured through-and-through with a 26-gauge needle (BD, Drogheda, Ireland). A small amount of stool was extruded to ensure wound patency. The cecum was replaced and the abdomen was closed using Sofsilk 6–0 (Ethicon). This model results in a marked septic response and death in 0–10% of animals. Sham animals underwent an identical laparotomy; the cecum was exposed but not ligated or punctured and replaced. All mice were administered 1 ml of sterile saline subcutaneously for fluid resuscitation post-operatively and 500 μl saline twice daily thereafter, containing 0.05 mg/kg buprenorphine. Nine and eight mice were included in the CLP or sham group per time point, respectively.

Induction of VRE peritonitis

Forty-eight hours after surgery, VRE peritonitis was induced as previously described [18]. Mice were injected intraperitoneally with approximately 10⁸ colony-forming units (CFU) of VRE in 200 μl sterile saline. Mice were killed 2 h, 1, 2 and 5 days thereafter.

Bacterial strain

VRE strain, E155, was used in all experiments. This clinical isolate from the Cook County Hospital, Chicago, IL, belongs to a genetic subpopulation of hospital-associated E. faecium that is responsible for the worldwide emergence of nosocomial multiresistant E. faecium, characterized by high-level quinolon and ampicillin resistance, a pathogenicity island, containing the variant esp gene, and the presence of five cell surface protein genes [20, 21].

Collection of samples

Mice were anesthetized by inhalation of isoflurane (Abbot, Laboratories Ltd., Kent, UK)/O₂ (2%/2 l), a peritoneal lavage was performed with 5 ml sterile phosphate-buffered-saline using a 18-gauge needle; peritoneal lavage fluid (PLF) was collected in sterile polypropylene tubes (BD, Breda, The Netherlands). Blood was drawn by cardiac puncture, transferred to heparin-gel vacutainer tubes and placed on ice. Liver and lungs were harvested. PLF supernatants and plasma were stored at −20°C until further examination.

Determination of bacterial outgrowth

The number of VRE CFU was determined in PLF, blood, liver and lung homogenates. The organs were homogenized in four volumes of saline and serial tenfold dilutions were made of the homogenates, PLF and blood, then 50 μl of each dilution was plated. Samples were plated on Slanetz-Bertley (SB) agar plates (Oxoid, Badhoevedorp, The Netherlands), supplemented with vancomycin (6 μg/ml), to determine the amount of VRE. No bacterial growth was observed when fecal pellets of naive mice were plated on these plates. Additionally, samples were plated onto BA, MacConkey (McC) (Difco, Detroit, MI) and colistin nalidixic acid (CNA) agar (BD, Breda, The Netherlands) for quantification of total aerobic, gram-negative and gram-positive bacteria, respectively. The plates were incubated at 37°C under 5% CO₂, and CFU were counted after 20 (BA and McC) or 44 (SB and CNA) hours.
Cell counts and differentials

Total cell numbers were counted from each PLF sample using a hemocytometer (Beckman Coulter, Fullerton, CA, USA). Differential cell counts were performed on cytospin preparations, stained with Giemsa (Diff-Quick; Dade Behring), by an investigator blinded for experimental groups.

Assays

Macrophage inflammatory protein (MIP)-2, cytokine-induced neutrophil chemoattractant (KC) and LPS-induced C-X-C chemokine (LIX) were measured in PLF by ELISA’s (R&D Systems, Minneapolis, MN) according to the manufacturers’ instructions. Tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, IL-12p70, interferon (IFN)-γ and monocyte chemoattractant protein (MCP)-1 were measured in PLF and plasma by using a commercially available cytometric bead array (CBA) multiplex assay (BD Biosciences, San Jose, CA, USA) in accordance with the manufacturer’s recommendations. Aspartate aminotransferase (ASAT) and creatinine were determined in plasma with commercially available kits (Sigma-Aldrich, St. Louis, MO, USA), using a Hitachi analyzer (Boehringer Mannheim, Mannheim, Germany).

Organ pathology

Livers and lungs were fixed in formaldehyde and embedded in paraffin for histological examination. Sections of 4 μm were stained with hematoxylin-eosin, and analyzed by a pathologist blinded for groups. To score liver injury, interstitial inflammation, thrombi, hepatocellular necrosis and portal inflammation were analyzed. Lungs were scored for interstitial inflammation, edema, pleuritis and thrombi [22].

Statistical analysis

Data are expressed as mean ± SEM. Serial data were analyzed by two-way analysis of variance (ANOVA) followed by a post hoc Bonferroni test. Two group comparisons were done by Mann-Whitney U test. GraphPad Prism version 4 (GraphPad Software, San Diego, CA, USA) was used. A P-value <0.05 was considered statistically significant.

Results

CLP strongly impairs clearance of VRE

To obtain insight into the impact of abdominal sepsis on host defense against VRE, mice were intraperitoneally...
injected with a non-lethal amount of VRE (10⁸ CFU) or saline at 48 h after CLP or sham surgery. CLP was associated with a strongly impaired clearance of VRE in all organs examined (Fig. 1). Sham mice showed a rapid decline in enterococcal loads from the peritoneal cavity; 2 days after infection VRE could not be recovered anymore from blood, liver or lungs and only 2/8 sham mice still had VRE in their peritoneal cavity. In contrast, in CLP mice enterococcal loads remained high in all body compartments examined for up to 5 days; 2 days after infection VRE loads were >1,000-fold higher in PLF, blood, liver and lungs of mice that had undergone CLP.

CLP results in a diminished neutrophil influx in response to VRE peritonitis

Peritoneal leukocyte counts and differentials did not differ significantly between CLP and sham mice 48 h after the procedure (directly before infection with VRE) (Fig. 2a, b). Sham mice demonstrated a strong and rapid increase in neutrophil counts in PLF peaking 2 h after infection with VRE. In contrast, CLP mice did not respond with an influx of neutrophils to the primary site of VRE infection, and neutrophil counts remained indistinguishable from CLP mice not infected with VRE (Fig. 2a). Peritoneal neutrophil numbers increased in all CLP mice later on irrespective of VRE infection, as a consequence of the CLP induced polymicrobial peritonitis [23]. After infection with VRE, both sham and CLP mice had an initial drop in macrophage numbers, followed by an increase after 24 h (Fig. 2b). CLP mice not infected with VRE had no significant change in macrophage numbers during this period. Sham mice injected with saline did not show any important changes in peritoneal cell numbers and differentials. CLP mice displayed highest numbers of neutrophils and macrophages at 48 h after VRE or saline injection (not shown). At 5 days peritoneal cells from all mice were back to naïve levels (not shown).

The murine CXC chemokines KC, MIP-2 and LIX are known neutrophil attracting and activating mediators. Sham mice responded with an increase in KC and LIX levels 2 h after VRE infection (Fig. 2c, d), whereas CLP mice subsequently infected did not. MIP-2 levels were low in all mice (not shown). Sham mice not infected with VRE showed low or undetectable chemokine levels throughout the entire experiment (not shown).

CLP causes an impaired peritoneal cytokine response to VRE peritonitis

At 48 h after CLP, directly before the administration of VRE, plasma and PLF concentrations of TNF-α, IL-6,
IL-10 and MCP-1 were similar to those measured 48 h after sham surgery, except for peritoneal IL-10 levels, which were higher after CLP (Fig. 3). The levels of these mediators remained low throughout the following 5 days in CLP mice not infected with VRE. Peritoneal IL-10 levels decreased from 24 h after VRE infection onward (not shown). Sham mice displayed an increase in peritoneal and plasma concentrations of TNF-α, IL-6, IL-10 and MCP-1 2 h after infection with VRE; the levels of these mediators rapidly decreased thereafter, returning to baseline levels at 24 h after infection, with the exception of peritoneal IL-10. At 2 and 5 days after infection, the local and systemic concentrations of all mediators measured were either very low or undetectable (not shown). Strikingly, in CLP mice peritoneal cytokine levels did not increase after induction of VRE peritonitis. Interestingly, CLP mice demonstrated a comparable increase in plasma TNF-α and IL-6 levels relative to sham mice after infection with VRE. Sham mice not infected with VRE showed low or undetectable cytokine levels throughout...
the experiment; IFN-γ and IL-12p70 levels were low or undetectable in any of the mice (not shown).

VRE peritonitis does not impact on host defense against CLP-induced sepsis

Superinfection with VRE did not result in increased CLP-induced lethality: during the 5-day observation period 6% of CLP mice with subsequent VRE infection died, whereas 8% of CLP mice with subsequent saline injection died. None of the sham mice died. Furthermore, no differences in weight loss were seen between CLP mice with or without VRE infection (not shown). CLP caused formation of an intra-abdominal abscess surrounding the ligated and perforated cecum, covered by small-bowel loops. No difference in the aspect of the abscess was seen after VRE infection compared to saline injection. No other abscesses were found in the abdominal cavity or organs up to 7 days after CLP. In addition, VRE infection did not influence the gram-negative or gram-positive bacterial loads in PLF, blood, liver or lungs after CLP (shown for total aerobic bacterial loads in fig. 4). Only mild inflammatory changes were seen in liver and lungs upon histopathological examination that did not differ between CLP mice with or without VRE infection (not shown). Nonetheless, CLP did induce hepatocellular injury and a reduced kidney function, as reflected by elevated plasma concentrations of ASAT and creatinine, respectively. However, ASAT and creatinine levels were comparable between CLP mice with or without VRE infection (Fig. 5). No pathology or altered ASAT or creatinine levels were seen in sham mice (not shown).

Discussion

In this study, we demonstrate that polymicrobial sepsis induced by CLP results in a strongly impaired peritoneal and systemic clearance of VRE, accompanied by a profoundly reduced early inflammatory response at the primary site of VRE infection. Although CLP mice infected with VRE had high enterococcal loads in multiple body sites for days, these animals did not show increased organ damage when compared to CLP mice not infected with VRE, as determined by histopathology of lung and liver tissue and laboratory markers for liver and kidney injury. These data suggest that infection and dissemination of VRE is greatly facilitated by sublethal polymicrobial sepsis, but that—at least in this model—VRE does not contribute to sepsis induced pathology to a significant extent.

Infections with *E. faecium* are a major problem in patients on intensive care units, and are associated with increased morbidity and mortality [24–26]. These infections are of special concern as they are increasingly difficult to treat due to the escalating antimicrobial resistance expressed by *E. faecium* [27, 28]. The aforementioned patient groups frequently suffer a period of

![Fig. 4 Enterococcus faecium infection does not influence total aerobic bacterial loads after CLP. Nine mice per time point underwent CLP and received $10^8$ CFU *E. faecium* (closed circles) or saline (open circles) intraperitoneally 48 h thereafter. Two hours, 1, 2 and 5 days after the second challenge mice were killed. Mean (±SEM) CFU of total aerobic bacteria in (a) peritoneal lavage fluid (PLF), (b) blood, (c) liver, and (d) lung are shown. During the 5 days of the experiment 6% of the mice with subsequent VRE infection died, whereas 8% of the mice with subsequent saline injection died. No differences in aerobic outgrowth were seen between groups](image-url)
sepsis during their hospital stay. Sepsis has been associated with a vigorous proinflammatory response, which contributes to the multisystem organ failure and mortality [8, 9]. Additionally, septic insults initiate concurrent or sequential anti-inflammatory cascades, which are important to avoid detrimental inflammatory responses and achieve immunological homeostasis. The anti-inflammatory response has been implicated in the inability of critically ill patients to eradicate the primary infection responsible for sepsis and their propensity to acquire secondary nosocomial infections [6–11].

CLP is considered a clinically relevant model for sepsis since it mimics a common clinical scenario and is associated with an early hyper-inflammatory reaction followed by a subsequent hypo-inflammatory phase [12, 29, 30]. Several studies have documented a reduced capacity to release cytokines after CLP and a subsequent challenge with bacterial antigens [23, 30, 31]. This sepsis-induced immune hypo responsiveness was shown to impair host defense mechanisms in the lungs against Pseudomonas aeruginosa and Streptococcus pneumoniae [13–15]. We here used the CLP model of sepsis to study the course of VRE peritonitis in a host with severe co-morbid disease.

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Sham operated control mice demonstrated an early inflammatory response and a rapid clearance of VRE similar to what we reported earlier in previously healthy mice that had not been subjected to abdominal surgery [18]. Hence, abdominal surgery per se does not appear to have a major impact on host defense against VRE, at least in this model.

Experimental studies have suggested that E. faecalis can influence abscess formation [35]. In our study we were unable to demonstrate that prolonged infection with VRE influences abscess formation, morbidity, mortality or organ damage in mice subjected to CLP. In addition, VRE infection did not impact on bacterial burdens associated with CLP. As such, our data argue against a causal role for VRE in adverse clinical outcomes in patients infected with this bacterium. We have to mention, however, that the mice used were healthy before going into surgery and 10 weeks of age resembling a situation of mid- to late-adolescence in humans. Additionally, the intensity of polymicrobial sepsis in this CLP model was relatively mild. It would be of interest to examine the impact of VRE infections in other models with diverse underlying illnesses, e.g., by using older male/female mice, being in an immunocompromised state before performing surgery.

However, our results clearly show that the host suffering from sublethal polymicrobial sepsis induced by CLP is severely impaired in clearing abdominal and systemic VRE infection, most likely due to an incapacity...
to respond to this bacterium at the primary site of infection.

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