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Effects of aging on the immunopathological response to sepsis

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Abstract

Objective—Aging is associated with increased inflammation following sepsis. The purpose of this study was to determine if this represents a fundamental age-based difference in the host response or is secondary to the increased mortality seen in aged hosts.

Design—Prospective, randomized controlled study.

Setting—Animal laboratory in a university medical center.

Subjects—Young (6–12 week) and aged (20–24 month) FVB/N mice.

Interventions—Mice were subjected to 2×25 or 1×30 cecal ligation and puncture (CLP).

Measurements and Main Results—Survival was similar in young mice subjected to 2×25 CLP and aged mice subjected to 1×30 CLP ($p=0.15$). Young mice subjected to 1×30 CLP had improved survival compared to both other groups ($p<0.05$). When injury was held constant but mortality was greater, both systemic and peritoneal levels of TNF- α , IL-6, IL-10 and MCP-1 were elevated 24 hours after CLP in aged animals compared to young animals ($p<0.05$). When mortality was similar but injury severity was different, there were no significant differences in systemic cytokines between aged mice and young mice. In contrast, peritoneal levels of TNF- α , IL-6, and IL-10 were higher in aged mice subjected to 1×30 CLP than young mice subjected to 2×25 CLP despite their similar mortalities ($p<0.05$). There were no significant differences in either bacteremia or peritoneal cultures when animals of different ages sustained similar injuries or had different injuries with similar mortalities.

Conclusions—Aged mice are more likely to die from sepsis than young mice when subjected to an equivalent insult, and this is associated with increases in both systemic and local inflammation. There is an exaggerated local but not systemic inflammatory response in aged mice compared to young mice when mortality is similar. This suggests that systemic processes that culminate in death may be age-independent, but the local inflammatory response may be greater with aging.

Keywords

Aging; CLP; Sepsis; Apoptosis; Cytokines; Survival

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INTRODUCTION

Sepsis is primarily a disease of the aged. Despite the fact that elderly patients account for 12% of the United States' population, over 60% of cases of sepsis occur in patients greater than 65 years of age, and 80% of deaths from sepsis occur in this age group (1;2). The average age of patients with sepsis has been increasing over time, and age is an independent predictor of mortality in septic patients (2;3).

Animal models suggest that the pathophysiology of sepsis is different in young and aged animals. When young and aged mice are subjected to either cecal ligation and puncture (CLP) or endotoxemia, aged mice have significantly increased mortality (4–8). Compared to young mice, aged mice subjected to CLP or endotoxemia also have increased systemic levels of inflammatory cytokines (4;6–8) and increased splenic apoptosis (9), both metrics associated with increased mortality in sepsis in human (10;11) and animal studies (12;13). Additionally, aged animals subjected to CLP are resistant to antibiotic therapy (4) and have increased pulmonary inflammation after either burn injury or endotoxemia (14;15).

While sepsis is more lethal in an aged population, it is not clear why the host response is age-dependent. One possibility is that aged mice have a fundamentally different pathophysiologic response to sepsis than young mice. This is relevant to critical care research in light of the fact that young mice are nearly always used as surrogates for aged patients in pre-clinical sepsis trials. If the accumulation of life's stresses and responses lead to reprogramming of the host response, it could explain why laboratory experiments conducted with young mice fail to translate into successful clinical trials in patients (16). Alternatively, the measured differences in the response to sepsis in young and aged animals may be secondary to the significantly increased mortality seen in aged vs. young animals subjected to the same insult. Remick et al have shown that the more likely an animal is to die from sepsis, the more pronounced its pro-inflammatory response will be (17). However, the association between mortality and pro-inflammatory response has been described in the setting of young mice subjected to increasing injury severity. In order to distinguish whether the age-dependent host response to sepsis is a function of a fundamental age-based difference or is secondary to increased mortality in aged mice, we calibrated injury severity in CLP to produce similar mortality in young and aged mice, and then studied local and systemic inflammatory responses.

MATERIALS AND METHODS

Sepsis model

Experiments were performed on young and aged gender-matched FVB/N mice (born and raised at Washington University in a barrier facility). Young and aged mice were defined as 6–12 week old and 20–24 month old respectively (4). Of note, unmanipulated FVB/N mice have previously been demonstrated to have a 60% survival rate at the age of 24 months (18).

CLP was performed by the methods of Baker et al (19). Briefly, anesthesia was induced 5% halothane and maintained with 2% halothane. A small midline abdominal incision was made, and the cecum exteriorized and ligated with 4–0 silk immediately distal to the ileocecal valve without causing intestinal obstruction. The cecum was then punctured once or twice with either a 25 or 30 gauge needle, and a small amount of stool was extruded (double puncture with a 25 gauge needle is referred to hereafter as 2 × 25 CLP, single puncture with a 30 gauge needle is referred to as 1×30 CLP). The cecum was replaced in the abdomen, and the abdomen was closed in layers. Mice received 1 mL 0.9% NaCl subcutaneously immediately post-operatively to account for insensible losses and received Buprenex, 0.1 mg/kg post-operatively for pain control. Metronidazole (35 mg/kg, Sigma, St. Louis, MO) and ceftriaxone (50 mg/kg, Sigma) treatment was initiated 1 hour after CLP and repeated every 12 hours for 2 days. Mice were

maintained on a 12 hour light-dark schedule in a specific pathogen-free environment and had unlimited access to standard laboratory mouse chow *ad libitum*. All animals were either sacrificed 24 hours after CLP or were followed 7 days for survival.

Historical controls in our laboratory demonstrated a 44% seven-day survival in young mice subjected to 2×25 CLP (20), whereas a similar insult attempted in pilot experiments for this manuscript was uniformly fatal within three days in aged animals (unpublished observations). Based upon those results, the next set of survival experiments (see results) used young mice subjected to 2×25 CLP and aged mice subjected to 1×30 CLP. All experiments were conducted in accordance with the National Institute of Health guidelines for the use of laboratory animals following approval by the Washington University Animal Studies Committee.

Cytokine determinations and cultures

Cytokine levels in peritoneal fluid and serum in both unmanipulated and septic animals were determined using a cytometric bead array (BD mouse inflammation kit, San Jose, CA) following manufacturers protocol. All animals were handled identically except unmanipulated animals did not undergo CLP prior to sacrifice. In septic animals, twenty four hours after CLP, mice were anesthetized with 43 mg/kg ketamine, 9 mg/kg xylazine and 1 mg/kg acepromazine. The peritoneal cavity was lavaged with 5 mL of 0.9% NaCl. Blood was then removed from the inferior vena cava for cytokine determination and cultures after which animals were exsanguinated while still under anesthesia. Serum was separated from whole blood using serum isolation tubes spun at 10,000 rpm for 10 minutes.

Whole blood and peritoneal fluid taken from the same septic animals were also serially diluted in 0.9% NaCl and plated on sheep's blood agar. Plates were incubated overnight at 37°C and the number of colonies enumerated.

Splenic apoptosis

Spleens were removed at the time of sacrifice, fixed in 10% formalin for 24 hours, embedded in paraffin, and stained for active caspase 3 (9). Sections were rehydrated then incubated at room temperature in 3% H₂O₂ in methanol for 10 minutes. They were then heated in Antigen Decloaking Solution (Biocare Medical, Concord, CA) for 45 minutes at 100 °C followed by blocking in 20% Goat Serum (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. This was followed by incubation with polyclonal rabbit anti-active caspase 3 (1:100; Cell Signaling, Beverly, MA) overnight at 4 °C. Sections were then incubated with secondary biotinylated goat anti-rabbit antibody (1:200; Vector Laboratories) and then Vectastain ABC (Vector Laboratories), both at room temperature for 30 minutes. Slides were developed with metal-enhanced diaminobenzidine solution and counterstained with hematoxylin and dehydrated. The number of active caspase 3 positive cells in 5 random high-power fields (400x) was then counted by an observer blinded to tissue identity.

Statistics

A statistician (DJD) performed all statistical analyses. Survival curves were compared using the log-rank test. Continuous data sets for cytokines, cultures, and apoptosis were tested for Gaussian distribution using the Shapiro-Wilk Normality Test and found to have non-Gaussian distributions. Therefore, the Kruskal-Wallis non-parametric one-way analysis of variance by ranks was used, followed by post hoc pairwise comparisons using the Wilcoxon Two-Sample Test for all comparisons. Data were analyzed using the statistical program SAS version 9.1 (SAS, Cary, NC). A p value <0.05 was considered to be statistically significant.

RESULTS

Effects of injury and age on mortality

To understand the impact of aging on the pathophysiology of sepsis independent of mortality, it was first necessary to establish a model of CLP where mortality was similar between young and aged animals. Survival was similar between young mice subjected to 2×25 CLP and aged mice subjected to 1×30 CLP (51% vs. 35%, $p=0.15$, Figure 1). By comparison, young mice subjected to 1×30 CLP had 73% survival ($p=0.03$ compared to young 2×25, $p=0.002$ compared to aged 1×30, Figure 1). All further experiments were done on these three groups of animals to compare the physiologic state of young and aged animals when a) mortality was varied but injury was held constant (young vs. aged 1×30) and b) mortality was similar but injury varied (young 2×25 vs. aged 1×30). A comparison of young animals with differing injuries and mortalities (young 1×30 vs. young 2×25) was also performed.

Systemic and local inflammatory response

Plasma and peritoneal levels of TNF- α , IL-6, IL-10 and MCP-1 were at or below the lower limit of detection in unmanipulated young and aged animals ($n=6$ /group, data not shown). However, when injury was similar (1×30 CLP), plasma levels of these cytokines were all markedly increased in aged septic mice compared to young septic mice ($p=0.002$ for TNF- α , $p=0.0006$ for IL-6, $p=0.003$ for IL-10, $p=0.004$ for MCP-1, Figure 2). In contrast, cytokine levels were not significantly different between young and aged septic mice in experimental models where they had similar mortalities but different magnitude of inciting injury (i.e. 2×25 vs. 1×30, $p=0.75$ for TNF- α , $p=0.51$ for IL-6, $p=0.21$ for IL-10, $p=0.11$ for MCP-1). Young animals subjected to a 2×25 CLP had increased systemic cytokines compared to young animals subjected to 1×30 CLP, consistent with previous reports demonstrating that severity of injury correlates with severity of the inflammatory response (17). Of note, neither IFN- γ nor IL-12 was detectable in the plasma in any septic animal.

Cytokines were also measured in the peritoneal fluid following CLP to determine if the local inflammatory response was similar to the systemic inflammatory response in young and aged animals. Similar to the systemic response, when injury was held constant, peritoneal cytokine levels of TNF- α , IL-6, and MCP-1 were generally increased in aged septic mice compared to young septic mice ($p=0.003$ for TNF- α , $p=0.02$ for IL-6, $p=0.10$ for IL-10, $p=0.03$ for MCP-1, Figure 3). In contrast to systemic cytokines, peritoneal cytokine levels were generally lower in young septic mice compared to aged septic mice with similar mortalities ($p=0.03$ for TNF- α , $p=0.04$ for IL-6, $p=0.001$ for IL-10, $p=0.28$ for MCP-1).

Systemic and local bacterial control

When animals of different ages sustained similar (1×30 CLP) injuries, there were no significant differences in bacteremia between young and aged animals ($p=0.20$, Figure 4A). When mortality was similar, despite the fact the young mice subjected to 2×25 CLP had a significantly more severe injury than aged mice subjected to 1×30 CLP, there were also no significant differences in bacteremia ($p=0.37$, Figure 4A). In contrast, young animals subjected to 2×25 CLP had an increased circulating bacterial load compared to young animals subjected to 1×30 CLP ($p=0.02$), consistent with their more significant injury and higher level of mortality.

Peritoneal bacterial burden was similar in young (2×25, 1×30) and aged (1×30) animals regardless of whether injury or mortality was matched ($p=0.40$, $p=0.68$ respectively, Figure 4B).

Splenic Apoptosis

When animals of different ages sustained similar (1×30 CLP) injuries, splenic apoptosis was increased in aged septic mice compared to young septic mice ($p=0.02$, Figure 5). In contrast, levels of splenic apoptosis were not significantly different between young and aged septic animals when mortality was similar ($p=0.38$). Young animals subjected to 2×25 CLP had increased splenic apoptosis compared to young animals subjected to 1×30 CLP, consistent with their more significant injury and higher level of mortality ($p=0.005$).

DISCUSSION

When injury was held constant between aged and young mice with a wide variation in 7-day survival, aged mice had a markedly increased systemic and local inflammatory response as well as markedly increased splenic apoptosis. When 7-day mortality was similar between aged and young mice, aged animals generally mounted a similar systemic inflammatory response, had similar levels of splenic apoptosis, and developed a similar degree of bacteremia despite receiving a much less severe CLP injury. In contrast, when 7-day mortality was similar between aged and young animals, aged animals mounted an exaggerated local inflammatory response although this did not result in improved local bacterial clearance. Taken, together, these data demonstrate that mortality of sepsis correlates with systemic cytokine levels (a proxy for inflammation) and that this relationship is independent of age. However, the local host response appears to be age-dependent.

These data are consistent with prior studies, showing that aged mice mount an exaggerated response compared to young mice following similar injuries (4;6;7). However when mortality is similar, there was no significant difference in circulating inflammatory cytokine levels between young and aged mice. Our results do not clearly illuminate why aged animals are more likely to die than younger animals subjected to the same insult. It is possible that susceptibility to mortality rather than age as an independent variable is the critical determinant of the immunopathological response to sepsis (animals more likely to die will have a more pronounced response). It is equally plausible that an animal's immunopathological response to sepsis determines its mortality (an animal with a more pronounced response is more likely to die).

Our results yield insights into the role aging plays in controlling infection. As expected, young mice with a more significant CLP injury (2×25) had increased levels of bacteremia than young mice with a smaller injury (1×30), consistent with an increased fecal inoculum from the larger cecal puncture. It should be noted that the concentration of bacteria found in the systemic circulation is substantially lower than a previous study that examined this parameter after CLP in young and aged mice (5). However, our study used a different strain of mice (FVB/N vs. C57Bl/6) and the injury was substantially less severe in our study (all animals had a 2×20 CLP in the prior study and almost all were dead within two days) so making direct comparisons between them is difficult. In contrast, aged mice with a minor CLP injury (1×30) had similar levels of peritoneal and systemic bacteria as young mice with a major CLP injury (2×25). This suggests a defect in bacterial clearance in the aged animals since it might have been predicted that animals with a smaller injury would have less bacteria in their peritoneal space or their bloodstreams in light of previous studies showing that innate immunity appears to be well preserved even at extremes of age (21). This is especially striking in the peritoneal cavity where there were similar concentrations of peritoneal bacteria despite a) an exaggerated local host response in aged animals and b) a smaller fecal inoculum due to the smaller CLP injury. The local response was accompanied by a greater than 2-fold increase in local IL-10 production by aged mice subjected to 1×30 CLP compared to young mice receiving a more severe insult. Although we are unaware of any data on IL-10 production in peritoneal fluid following CLP in aged animals, this is consistent with data demonstrating that aging induces elevated

splenocyte production of IL-10 following burn injury (22) and local heart and lung mRNA expression of IL-10 in sepsis (6). While tissue-specific IL-10 has been demonstrated to improve survival in young animals in sepsis (23–25), the biological significance of the difference in peritoneal IL-10 in this study is unclear. Multiple possibilities may account for the relative defect in bacterial clearance in aged mice. These include a subtle defect in neutrophil trafficking in the peritoneal cavity, delayed bacterial opsonization, an impaired circulatory response secondarily affecting local inflammation, or the presence of other humoral or cellular factors causing impaired bacterial clearance. Examining these possibilities is beyond the scope of this paper, but is a line of investigation we believe should be pursued in the future. Additionally, the gut's endogenous microflora has been shown to play a major role in critical illness (26; 27) and aging has been demonstrated to alter the intestinal microflora with an increase in enterobacteria and a decrease in anaerobes and bifidobacteria (28). It would therefore be reasonable to examine both the gut and the virulence of the bacteria released following CLP as potential reasons for why mortality is higher in aged animals subjected to the same insult.

This study has several limitations. First, it examines animals only at a single timepoint. While we chose this timepoint based on multiple studies demonstrating significant differences in the host response to sepsis between young and aged animals 24 hours after CLP (4–6;9;29), the results represent a “snapshot” view and cannot provide any information as to the kinetics of the cytokine levels, microbial burden, or splenic apoptosis. This is especially relevant in light of a recent publication demonstrating that late deaths in experimental sepsis are associated with a different immunopathologic profile than early deaths (30). Next, we did not examine functional immunity, but rather used systemic and local cytokines as a surrogate for immune function, which may give an incomplete or inaccurate assessment of the immune response at the cellular level. For instance, it has been demonstrated that the adaptive immune response is altered in aged patients with a skewing towards a TH2 phenotype (21), but we did not study the TH1/TH2 phenotype in this study. Next, the interpretation of our results is based upon our conclusion that mortality is similar between aged animals subjected to a 1×30 CLP and young animals subjected to a 2×25 CLP. This is based upon a p value of 0.15 between the groups and the acceptance of a statistical difference between groups only for a p value <0.05. However, since there was a significant disparity in the number of animals in each group on the survival curve (17 and 47 respectively), it is possible that if more animals were added to the aged 1×30 group, we might have uncovered a statistically significant difference in mortality which would then alter the interpretation of our results. Similarly, having a p value >0.05 does not necessarily mean that there is no true difference between the groups.

The use of an approximately 50% mortality study design also means that the animals assayed at 24 hours for cytokines, cultures, and apoptosis include a mixture of those that would ultimately have survived their injury and those that would have succumbed to it. Since the pathophysiology may be different between survivors and nonsurvivors, it is possible that important readouts may have been missed by our study design which might have been better observed in a CLP model in which all animals died. Additionally since only 90% of mice survived to 24 hours, surveying cytokines, cultures, and apoptosis at this timepoint is selecting for animals that survived to this timepoint and may miss important information that would have been obtained if animals were assessed at 6 or 12 hours.

Both male and female mice were used in this study. While animals in all experiments were gender-matched, female mice have a clear survival advantage compared to male mice (31; 32) following CLP. It is therefore possible that by combining genders in our results we are obscuring important gender-based differences in young and aged animals that occurred despite the similar number of males and females in experiments. Additionally, the number of bacteria in peritoneal cultures may vary depending on the nature and organization of the inflammatory response induced by fecal contamination following CLP. Cultures of solid organs such as the

liver or spleen may have yielded a less variable assessment of intraabdominal bacterial burden. Next, we do not actually know why animals are dying after CLP. There is evidence that septic animals have multi organ failure, and we believe that the ability to wall off an abscess is associated with survival (i.e. animals with fecal peritonitis that is not contained are more likely to die than those that form an organized collection). However, our results do not definitively delineate the cause of death after CLP. Finally, we have limited our studies to a single strain of mice. Since both lifespan and genetic susceptibility to sepsis vary between mouse strains (33), it is unclear how generalizable our results are.

Despite these limitations, our results demonstrate that there is a striking parallelism in the systemic immunoinflammatory response to sepsis in young and aged mice when mortality is similar. Although aged mice have a significantly increased inflammatory response compared to young mice subjected to the same injury, there is little difference in systemic cytokine response, bacterial burden or splenocyte apoptosis when mortality is matched. In contrast, there appears to be an exaggerated local immunoinflammatory response to sepsis in aged mice, although this is not associated with enhanced bacterial clearance. Together, these results suggest that some of the pathways of sepsis after CLP in aged mice are parallel to those in young mice (although the threshold for an insult to induce dysregulated septic immune response is significantly lower) but that some elements of the host response are fundamentally age-dependent.

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Figure 1.

Survival in young and aged septic mice. Survival was similar between young mice subjected to 2×25 CLP and aged mice subjected to 1×30 CLP. In contrast, young mice subjected to 1×30 CLP had improved survival compared to both of the other two groups of animals (* signifies $p < 0.05$ for both). All animals were treated with fluid resuscitation following CLP and received two days of antibiotics post-operatively.

Figure 2.

Plasma cytokine levels 24 hours after CLP. Cytokine levels were statistically higher in aged mice subjected to the same insult (1x30) than young mice. However, when mortality was similar (aged 1x30 vs. young 2x25), cytokine levels were not statistically different. Data shown are mean $\pm$ standard error of the mean.

Figure 3.

Peritoneal cytokine levels 24 hours after CLP. Cytokine levels were statistically higher in aged mice subjected to the same insult (1x30) than young mice for all except IL-10. When mortality was similar (aged 1x30 vs. young 2x25), all cytokine levels except MCP-1 were statistically higher in aged mice than in young mice. Data shown are mean $\pm$ standard error of the mean.

Figure 4.

Blood and peritoneal bacterial cultures 24 hours after CLP. Systemic (A) and peritoneal (B) cultures showed similar amounts of bacteria between young and aged mice subjected to the same insult (1×30) and between young and aged mice with similar mortalities despite the differences in the size of the fecal inoculum (2×25 vs. 1×30). Data were log transformed and are shown as mean ± standard error of the mean.

Figure 5.

Splenic apoptosis 24 hours after CLP. Apoptosis is statistically higher in aged mice subjected to the same insult (1x30) than young mice (A). However, when mortality is similar (aged 1x30 vs. young 2x25), cytokine levels are not statistically different. Data shown are mean $\pm$ standard error of the mean. Representative sections stained for active caspase 3 (positive cells are brown) are shown for young 1x30 (B), aged 1x30 (C) and young 2x25 (D), magnification 400x.