Neutrophil C5a receptor and the outcome in a rat model of sepsis[1]

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SPECIFIC AIMS

Signaling in neutrophils involving the powerful complement anaphylatoxin (C5a) and its receptor (C5aR) plays an essential role in innate immunity. We have previously shown in rodents that blockade of either C5a or C5aR dramatically improves survival rates in experimental sepsis. We have also shown that defensive functions of neutrophils are compromised during sepsis in a C5a-dependent manner. The main aim of the current study was to elucidate the mechanisms for C5aR alteration on neutrophils during sepsis and to evaluate the relationship between C5aR levels on neutrophils and survival after the onset of sepsis.

PRINCIPAL FINDINGS

1. Loss of C5aR content on blood neutrophils during sepsis

To understand the dynamics of C5aR expression on neutrophils during sepsis, C5aR content on blood neutrophils of rats was quantitatively evaluated by flow cytometric analysis 0, 4, 12, 24, 36, and 48 h after the onset of cecal ligation/puncture (CLP) -induced sepsis. Blood neutrophils from control animals showed a positive staining with anti-C5aR, while very limited staining was seen with preimmune serum (**Fig. 1*A***). During experimental sepsis, C5aR content on blood neutrophils was sharply decreased as early as 4 h after onset of CLP, reaching a nadir at 24 h. The intensity of C5aR staining was almost as low as the staining found with preimmune serum, suggesting a very low level of C5aR expression on neutrophils at this time. C5aR levels increased progressively thereafter (Fig. 1*A*).

2. C5a produced during sepsis causes the loss of C5aR content on neutrophils

To address whether excessive C5a generated during sepsis contributed to the loss of C5aR content on neutrophils, systemic C5a was blocked by an intravenous infusion of 500 μg anti-C5a rabbit IgG given immediately after the onset of sepsis. A companion group of CLP rats was similarly treated with 500 μg preimmune IgG. C5aR content on neutrophils was analyzed 12 h after CLP. Anti-C5a treatment substantially preserved C5aR content on neutrophils, resulting in a 65% increase ($P<0.05$) compared with preimmune IgG treatment (Fig. 1*B*).

3. Internalization and reconstitution of C5aR on neutrophils during sepsis

To determine the mechanism by which C5aR content on neutrophils is regulated during sepsis, we evaluated C5aR protein and mRNA levels in purified neutrophils 0, 24, and 48 h after CLP. Western blot analysis of whole cell lysis of rat neutrophils revealed a band with ~44 kDa position characteristic of rat C5aR. As determined by Western blot analysis, there were no significant changes in C5aR protein levels obtained from extracts of neutrophils isolated 0, 24, and 48 h after onset of sepsis (data not displayed). Concordantly, C5aR mRNA levels showed no differences. To visualize the C5aR translocation in neutrophils during sepsis, we conducted confocal fluorescence microscopy analysis. In control cells C5aR showed a uniform distribution in the cell periphery, indicating membrane staining. In contrast, a diffuse pattern of staining was found in the cytoplasmic compartment of 24 h CLP neutrophils, accompanied by disappearance of the peripheral staining indicative of C5aR internalization. In neutrophils 48 h after CLP, C5aR expression appeared to be in both the membrane and cytoplasm of cells, suggesting that C5aR recycling occurred. These data suggest that C5aR on neutrophils internalizes at an early stage of sepsis and is re-expressed later.

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[1] To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.03-0009fje; doi: 10.1096/fj.03-0009fje

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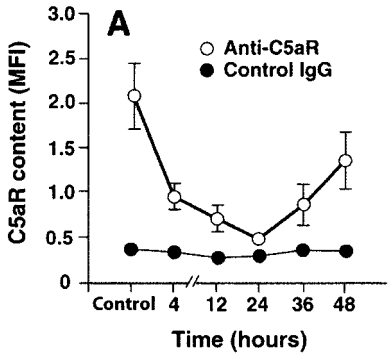

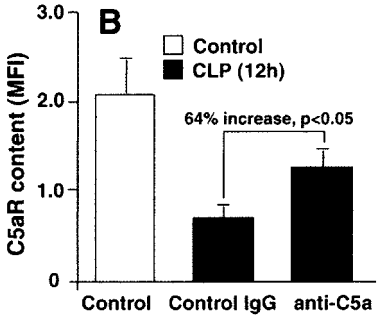

Figure 1. C5aR expression on neutrophils during experimental sepsis (*A*). Whole blood samples from controls (normal rats) and CLP animals were stained with anti-C5aR rabbit serum or preimmune serum, and C5aR content on blood neutrophils was evaluated by flow cytometric analysis at the times indicated. Effect of C5a blockade on C5aR expression in sepsis (*B*). C5a was blocked by an intravenous injection of 500 μg anti-C5a rabbit IgG immediately after the onset of CLP. Control animals were subjected to sham surgery. C5aR content on blood neutrophils was assessed 12 h after CLP. All values are mean ± SE (n=5).

4. Changes in functional responses of neutrophils are associated with altered C5aR levels on neutrophils

Control neutrophils and cells isolated from rats 24 and 48 h after CLP were chosen to represent the three different levels of C5aR expression. We first evaluated the effect of phorbol 12-myristate 13-acetate (PMA) on C5aR internalization, as PMA is a strong inducer of C5aR internalization. 100 nM PMA strongly diminished the C5aR staining on control neutrophils, and there was no further internalization observed when 24 h CLP cells were treated with PMA. However, a significant internalization in response to PMA was seen again on neutrophils from 48 h CLP rats. As expected, 10 nM C5a induced a vigorous in vitro chemotactic response for control neutrophils, with a chemotactic index (CI) of 9.05 ± 1.28. C5a-induced chemotaxis was severely impaired in cells from 24 h CLP animals (CI=2.62± 0.55), while chemotaxis ability was significantly improved in 48 h CLP cells compared with 24 h CLP cells (CI=4.95±1.34; P<0.05). Another manifestation of functional recovery for 48 h CLP neutrophils was obtained by assessing C5a-induced reactive oxygen species (ROS) production in these cells. 100 nM C5a evoked a marked increase in ROS generation in control cells. This effect was completely abolished in 24 h CLP cells. However, 48 h CLP cells appeared to produce a significant amount of ROS in response to C5a (P<0.05 vs. buffer control). These data indicate that alteration of C5aR content on neutrophils during sepsis is associated with functional responses of neutrophils.

5. C5aR reconstitution on neutrophils during sepsis correlates with the survival of septic animals

The data described above led us to speculate that C5aR reconstitution may be linked to the outcome in sepsis. To test this hypothesis, we assessed the survival of CLP rats and in parallel C5aR levels on blood neutrophils 36 h after CLP. 23 of 37 rats survived 36 h after CLP (**Fig. 2*A***). Blood samples were taken from the tail veins

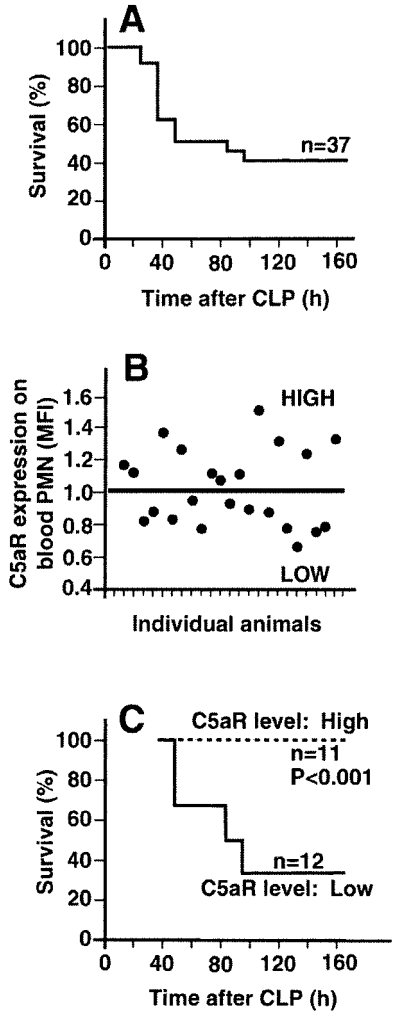

Figure 2. Correlation of C5aR levels on neutrophils with survival. 37 rats were subjected to CLP procedure and a survival curve was obtained over a 7 day interval (*A*). Blood samples were taken from tail veins 36 h after CLP, and C5aR levels were evaluated by flow cytometry analysis. Based on the median (*B*), the animals were divided into two groups: animals with neutrophil C5aR levels higher than median (HIGH) and animals with neutrophil C5aR levels lower than median (LOW). Death of individuals from these two groups was monitored (*C*).

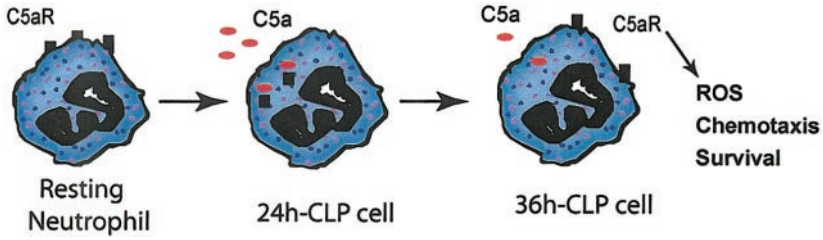

Figure 3. Schematic diagram showing dynamic changes in C5aR expression on neutrophils and ensuing functional outcomes during sepsis.

of survivors in order to assess C5aR levels on blood neutrophils. Based on the overall median value (1.03), we divided these animals into two groups: high levels (>1.03) of C5aR group (n=11) and low levels (<1.03) of C5aR group (n=12) (Fig. 2*B*). All animals with higher levels of C5aR survived (n=11; 100%), whereas only 4 of 12 (33%) animals with lower levels of C5aR survived during a 7-day interval (Fig. 2*C*). The difference between the outcome of these two groups was statistically significant (P =0.001 as assessed by proportional hazards modeling). Thus, the level of C5aR surface expression 36 h after onset of sepsis was positively correlated with the survival of the individual animals.

CONCLUSIONS

In this study, by using a clinically relevant rat model of sepsis, we were able to define the dynamic pattern of C5aR expression on neutrophils in vivo. C5aR on neutrophils internalized at early stage of sepsis (<24 h), and C5aR content on neutrophils began to be reconstituted thereafter (Fig. 1*A* and **Fig. 3**). The quick decrease of C5aR content on neutrophils early in sepsis was caused at least in part by excessive C5a generation in vivo, since blockade of C5a significantly preserved C5aR content on neutrophils. At a late stage of sepsis (>24 h), C5aR on septic neutrophils started to undergo a functional recovery, as reflected by increased content of C5aR, associated with enhanced ROS production as well as reconstitution of chemotactic responses to C5a. We also demonstrated that the level of C5aR re-expression on septic neutrophils from 36 h CLP animals was positively correlated with the survival of individual animals. Based on these findings, we postulate that higher levels of C5aR on neutrophils at late-stage sepsis are related to an efficient defense system for bacterial killing, resulting in survival during sepsis. Alternatively, it is possible that these animals that demonstrated more effective recovery of C5aR on neutrophils produced less C5a during sepsis. This study suggests that C5aR is a critical component in neutrophil action, including clearance of excessive C5a and fighting bacterial infection during sepsis. FJ