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Deficiency of complement factor C5 reduces early mortality but does not prevent organ damage in an animal model of multiple organ dysfunction syndrome

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Objective: To evaluate the role of complement factor C5 in a model of zymosan-induced multiple organ dysfunction syndrome.

Design: Experimental animal study.

Setting: Central animal laboratory of a university hospital.


Interventions: On day 0, all mice received an intraperitoneal injection with zymosan suspended in paraffin in a dose of 1 mg/g body weight.

Measurements and Main Results: Between days 0 and 12, biological parameters (temperature, body weight, and clinical condition) were measured daily and mortality was monitored. Clinical condition was assessed as a symptom score by blindly grading the degree of lethargy, conjunctivitis, diarrhea, and ruffled fur of each mouse on a 2-point scale (maximum score of 4). On day 12, all surviving mice were killed and relative organ weights of lungs, liver, spleen, and kidneys were calculated. Relative organ weight was defined as (organ weight/body weight) × 100%.

Zymosan administration induced a typical triphasic illness. Deterioration of the clinical condition, as indicated by the symptom score, and the decrease in temperature and body weight in the acute phase were all significantly less severe in C5-deficient mice (p < .005). In the late phase, no differences could be noticed in the courses of these biological parameters. Overall mortality was 2 (8%) of 25 in C5-deficient mice and 8 (32%) of 25 in C5-sufficient mice (p = .049), a difference that was mainly due to a difference in the acute phase. Organ damage, assessed as the relative organ weights, did not show any statistical differences for any organ between both strains.

Conclusions: Complement factor C5 appears to play an important role in the acute hyperdynamic septic response in this model. However, deficiency of C5 could not prevent organ damage in the late multiple organ dysfunction syndrome phase. This finding suggests that other factors must be more important in the development of the inflammatory response leading to multiple organ dysfunction syndrome. (Crit Care Med 1995; 23:1686–1693)

Key Words: complement 5a; disease models, animal; zymosan; mortality; adult respiratory distress syndrome; multiple organ failure; sepsis; inflammatory response; critical illness

Despite advances in intensive care treatment, multiple organ dysfunction syndrome and the adult respiratory distress syndrome (ARDS) still remain the most common causes of death in patients admitted to a surgical intensive care unit after major abdominal surgery, acute pancreatitis or severe trauma (1). It has been hypothesized that multiple organ dysfunction syndrome and ARDS could be the result of a generalized excessive autodestructive inflammatory response (2). Since the complement system is an important initiator and mediator of the inflammatory response (3), an excessive activation of the complement system has been implicated in the pathogenesis of multiple organ dysfunction syndrome and ARDS (4–7).
The complement system consists of at least 25 plasma proteins, formed by the liver and macrophages (4, 8–10). Two pathways of complement activation currently are recognized: the classical pathway, which is activated by antigen-antibody complexes (11), and the alternative pathway, which is activated by foreign material and tissue injury (12). Both pathways converge at the breakdown of C3, the subsequent cleavage of C5, and the activation of the final common pathway (3, 4). C5 is divided enzymatically into C5a and C5b. C5a is both an anaphylatoxin and a chemotactic agent. The latter function is probably the most important since its anaphylatoxic activity. Its chemotactic activity stimulates activation, aggregation and adherence of polymorphonuclear granulocytes to the endothelium with subsequent degranulation and release of oxygen radicals (13), vasoactive substances, and a variety of proteases which can cause endothelial damage (3, 14, 15), resulting in generalized edema. The chemotactic activity of C5a and C5a des Arg is specifically enhanced by Gc-globulin and is inactivated by chemotactic factor inactivator (16, 17). Pro-inflammatory cells possess a receptor for C5a (18). In addition to many inflammatory functions, C5a also has immunoregulatory activities. For example, C5a induces the release of interleukin (IL)-6 by stimulated monocytes (19–21) and stimulates the production of IL-1 after binding to macrophages (19, 22).

C5b participates together with C6, C7, C8, and C9 in the formation of the terminal complement complex. The terminal complement complex exists in two analogous forms. One form exists in a fluid phase in combination with the S-protein: this complex is nonlytic, and can be detected in plasma and inflammatory fluids (19, 22–24). The other form is the membrane attack complex, which can cause cell-lysis by penetrating lipid membranes such as the endothelium (25). Excessive complement activation can thus result in a generalized endothelial damage with a subsequent generalized permeability edema and organ injury (13).

We have developed a model of multiple organ dysfunction syndrome in which an intraperitoneal injection of zymosan suspended in paraffin leads to a generalized inflammatory response and histopathologic changes closely resembling the clinical entity of multiple organ dysfunction syndrome (26). This model has been validated in our laboratory and by others (27–30). Zymosan is a particulate cell wall product of the yeast, Saccharomyces cerevisiae, which contains, with some variations, 73% polysaccharides (mainly β-glucan and α-mannan), 15% proteins, 7% lipids, and inorganic components (31). Classically, zymosan has been identified as a potent activator of the alternative complement pathway (32). Unopsonized zymosan also stimulates macrophages to the release of various inflammatory mediators (33). The interaction between zymosan and macrophages appears to be mediated by three classes of cell surface receptors, namely, the mannose/fucose receptor (34), the β-glucan receptor (35), and the CR3 (CD11b/CD18) receptor (36).

In this model, interruption of complement activation could theoretically lead to an attenuation of the inflammatory response which leads to the development of multiple organ dysfunction syndrome.

In order to test this hypothesis, we used C5-deficient mice to evaluate the role of C5 in the development of multiple organ dysfunction syndrome.

MATERIALS AND METHODS

Zymosan. Zymosan A (Sigma Chemicals, St. Louis, MO) was irradiated with 5 kGy and suspended (2.5 g/100 mL) by high frequency vibration in liquid paraffin, for 60 mins at 40°C. This suspension was sterilized by incubation in a waterbath at 100°C for 80 mins. Sterility was confirmed by aerobic incubation on blood-agar plates for two days at 37°C. Before utilization, the zymosan was warmed to 40°C and vibrated in a high-frequency waterbath for 15 mins.

Animals. B10D2/Old.Sn and B10D2/New.Sn mice were obtained from Jackson Laboratories (Bar Harbor, ME) and from Harlan Olac Limited (Bicester, UK), respectively. B10D2/Old.Sn mice are congenitally C5-deficient by a deletion mutation on chromosome 2 (37). The B10D2/New.Sn mouse is the coisogentic twin of the B10D2/Old.Sn mouse, but without the mutation on chromosome 2 (37). C5 deficiency in B10D2/Old.Sn mice was shown to be accompanied by an almost complete absence of hemolytic complement activity (38). Only male mice were used, because female mice were reported not to be consistent in their C5 concentrations (39).

Throughout the experiment, all mice were allowed free access to water acidified with hydrochloric acid to a pH of 3 and were fed standard laboratory chow (RMH-GS pellets, Hope Farms, Woerden, The Netherlands) irradiated at 10 kGy. Room temperature was kept constant at 21°C and a 12-hr lighting cycle was maintained. The experiments
were approved by the Animal Care Committee of the Medical Faculty of Nijmegen.

Experimental Design. Twenty-five C5-deficient mice (B10D2/Old.Sn.), 14 wks old and weighing 19 to 32 g, and 25 control mice (B10D2/New.Sn.), 12 wks old and weighing 22 to 29 g, were adapted to handling 2 wks before the start of the experiment. On day 0 of the experiment, all mice received an aseptic intraperitoneal injection of zymosan in a dose of 1 mg/g body weight. Between days 0 and 12, body temperature, body weight, and clinical condition were measured and monitored daily. Clinical condition was assessed as a symptom score by grading the severity of conjunctivitis, diarrhea, ruffled fur, and lethargy in a blinded fashion on a 2-point scale (0 = none, 1 = present; minimum = 0, maximum = 4). Mortality rate was monitored daily. On day 12, all surviving mice were anesthetized with ether and bled by retro-ocular puncture. The abdomen was opened, using a sterile technique, and samples of the peritoneal fluid were collected for aerobic and anaerobic culture on blood-agar plates for 2 days at 37°C. Subsequently, lungs with trachea, spleen, liver, and kidneys were inspected, dissected free, and weighed.

Relative organ weights were calculated using the formula: (organ weight/body weight) × 100%. In mice that died prematurely before day 12, no anaerobic cultures were performed from the peritoneal fluid because post mortem anaerobic overgrowth could be expected.

Statistical Analysis. Statistical analysis of biological parameters (body temperature, body weight, and symptom score) was performed using the distribution-free curve analysis according to Koziol et al (40). Since the zymosan-induced illness is characterized by distinct phases, comparisons were made both for the acute phase (days 0 to 4), and the late phase (days 8 to 12). Relative organ weights were compared, using the distribution-free Wilcoxon's two sample test. Noncontinuous data (mortality rate) were analyzed, using Fisher's exact test. Differences between groups were considered to be statistically significant at \( p < .05 \).

RESULTS

Biological Measurements. Intraperitoneal administration of zymosan induced a typical triphasic clinical illness, depicted in Figure 1 as the course of the symptom scores. In the acute phase (days 0 to 4), the mice became lethargic and anorectic, hyperventilated, lost hemorrhagic fluids from the nose and conjunctivae, and had diarrhea. According to the symptom score, C5-deficient animals displayed significantly less severe (\( p < .0001 \)) symptoms than C5-sufficient mice. After 3 days, the condition of the surviving mice improved: they became more active, showed no signs of conjunctivitis or diarrhea, and their fur was only slightly ruffled. Recovery of C5-deficient mice appeared to be quicker. However, after 8 days, the clinical condition of the mice deteriorated progressively. They became more lethargic and started to lose hemorrhagic fluids from the nostrils and conjunctivae. C5-deficient mice deteriorated significantly slower (\( p < .0001 \)) than their C5 sufficient controls.

Figure 1 shows that body temperature declined dramatically in C5-sufficient mice the first day after zymosan injection. C5-deficient mice, however, showed little decrease (\( p < .001 \)) in temperature. After a recovery phase, temperature decreased again in the late phase. At this time no significant differences were noticed between the two groups.

The course of body weight was also triphasic, as shown in Figure 1. Body weight decreased in the acute phase. Recovery in C5-deficient mice was significantly better (\( p < .0001 \)) than in the C5-sufficient controls. After this recovery phase, body weight decreased again in both groups, with a less serious decrease in C5-deficient mice. However, this difference did not reach statistical significance (\( p = .0552 \)).

Survival Rates. Survival rates for both groups are depicted in Figure 1. In the acute phase, seven C5-sufficient mice died vs. one C5-deficient mouse. No mice died in the recovery phase. In the late phase, one mouse died in each group. Overall mortality was 8% (two of 25) in C5-deficient mice and 32% (8 of 25) in C5-sufficient controls (\( p = .049 \)), due mainly to the difference observed in the acute phase.

Macroscopic Appearance of the Organs and Relative Organ Weights. After samples of the peritoneal fluid had been taken for anaerobic and aerobic bacterial cultures, the organs were dissected free and inspected. The organs of the mice that died in the acute phase showed little abnormalities. The lungs were hyperemic and the abdomen showed few or no adhesions. No differences were noticed between groups.

The lungs of mice that died in the late phase or that were killed on day 12 were extremely hyperemic, with hemorrhagic spots, and occasionally, extensive hemorrhagic infarction. The abdomen showed signs of an extensive fibroplastic peritonitis with massive adhesions.

Although the C5-sufficient control mice showed the most extensive macroscopic abnormalities, no significant differences were found with the C5-deficient mice (data not shown).
Figure 1. A) Clinical condition of the surviving mice assessed as the symptom score by grading the severity of conjunctivitis, diarrhea, ruffled fur, and lethargy in a blinded fashion on a 2-point scale (0 = none, 1 = present; minimum = 0, maximum = 4). Complement factor C5-deficient mice showed significantly less symptoms in the acute and late phase (curve analysis according to Koziol). B) Body temperature of the surviving mice calculated as the percentage change when compared with day 0. In contrast to complement factor C5-sufficient controls, complement factor C5-deficient mice showed a significantly smaller decrease in temperature in the early phase. No significant differences were noticed in the late phase (curve analysis according to Koziol). C) Body weight of the surviving mice calculated as the percentage change when compared with day 0. Recovery in the early phase was significantly better in the complement factor C5-deficient mice. No significant differences were noticed in the late phase (curve analysis according to Koziol). D) Survival rate after injection of zymosan on day 0. Overall mortality rate was significantly lower (p = .049 by Fisher's exact test) in complement factor C5-deficient mice mainly due to a difference in the acute phase. In all graphs, the solid circles indicate complement factor C5-sufficient controls and the open circles indicate complement factor C5-deficient mice. Values in graphs A through C are expressed as mean ± SEM.

DISCUSSION

Both clinical and experimental data have suggested a relationship between activation of the complement system and the pathogenesis of septic shock, ARDS, and multiple organ dysfunction syndrome. During septic shock, most clinical studies have shown an activation of C5a. However, data are
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Figure 2. Relative organ weights 12 days after zymosan injection. Relative organ weights were calculated using the formula: (organ weight/body weight) × 100%. Solid bars, complement factor C5-sufficient controls; stippled bars, complement factor C5-deficient mice. No significant differences were noticed in terms of the organ damage of the surviving mice (Wilcoxon's two-sample test). Organ weights are expressed as percentage of body weight. Data are expressed as mean ± SEM.

conflicting when C5a is correlated to prognosis. Successful treatment of septic shock was associated with a normalization of the C5α/C5α des Arg ratio and terminal complement complex concentrations, but not of C5α concentrations (25, 41). Other studies (42, 43) could not confirm that the severity or mortality of sepsis is correlated with a persistent increase in C5α concentrations. On the other hand, a strong correlation was found between increased plasma concentrations of C5α and the development of ARDS (44), while C5α concentrations correlated with the severity of pulmonary failure (45). It also has been demonstrated that C5α is increased in bronchoalveolar lavage fluid in patients developing ARDS, with a normalization after ARDS had resolved (16). Chemotactic factor inactivator, which decreases C5α-directed neutrophil chemotaxis, has been shown to be markedly increased in ARDS bronchoalveolar lavage fluid. However, chemotactic factor inactivator-functional activity was markedly decreased, suggesting that patients with ARDS are functionally deficient in chemotactic factor inactivator, with a subsequent increased ability of C5α to attract neutrophils (46). Plasma terminal complement complex concentrations have been shown to be increased 2 days before the onset of ARDS in septic patients and to be decreased immediately after resolution, suggesting that terminal complement complex formation could be a good predictor of ARDS (47). Other studies (43, 48, 49) could not confirm a relationship between C5α or terminal complement complex formation and the development of ARDS or mortality from ARDS. Only a few studies have been conducted into the possible relationship between complement activation and the development of multiple organ dysfunction syndrome. Heideman and Hugli (5) reported a possible correlation between high concentrations of C3α and C4α, but not of C5α, with the development of multiple organ dysfunction syndrome. Nuytinck et al. (6) showed a good correlation between the multiple organ failure score and early C1q, C3, C3 proacti­

ator and C4 concentrations, but not with C5α concentrations. Roumen et al. (50) demonstrated recently in patients with severe blunt trauma that an early increase in C3α and terminal complement complex concentrations early after severe blunt trauma were associated with the development of multiple organ dysfunction syndrome. However, only C3α/C3 ratios early after trauma, and not terminal complement complex concentrations, were associated with mortality.

In most clinical studies, it is difficult to interpret measured concentrations of C5α since C5α, and to a lesser extent, C5α des Arg, are relatively unstable molecules and concentrations of C5α or C5α des Arg do not always reflect the level of C5α activation. C5α is rapidly cleared from the circulation and converted to C5α des Arg by serum carboxypeptidase. Furthermore, C5α des Arg is rapidly bound and removed from plasma by neutrophils (51). The conflicting results concerning the correlation between septic shock, ARDS, and multiple organ dysfunction syndrome, and the activation of C5α might be explained by this observation.

In order to find a possible causal relationship between complement activation and the development of septic shock, ARDS, and multiple organ dysfunction syndrome, experiments have been performed with complement depletion and, more specifically, C5 depletion by using C5-deficient animals or using antibodies against C5α. In a porcine endotoxic shock model (52), complement depletion with Naja haje cobra venom factor significantly improved cardiac index and visceral perfusion. In another porcine model (53) using a continuous infusion with *Pseudomonas aeruginosa*, complement depletion resulted in less pulmonary failure. In a primate model of sepsis with infusion of *Escherichia coli* (54), treatment before and during infusion with anti-C5α-antibodies resulted in a reduction of mortality, an attenuation of ARDS and decreases in the
systemic manifestations of sepsis. Peak C5a concentrations were decreased and no significant differences were seen in C3a or C4a concentrations (55). These experiments were confirmed by others in an endotoxic rat model (56) in which anti-C5a-antibodies attenuated the hypotensive and vascular permeability changes after endotoxin-induced shock. C5-deficient B10D2/new mice showed less septic lung injury and less early mortality in the cecal ligation and puncture model, although total mortality was equal to that in C5-sufficient B10D2/old mice (37). In another study (57), it was demonstrated that C5-deficient mice were protected from combined tumor necrosis factor-endotoxin-induced mortality, shock, hypothermia, hemoconcentration and bowel injury. The latter study (57) also showed the importance of C5 since C3-deficient mice were not protected.

The present study shows that C5-deficient mice displayed an attenuated response in the acute hyperdynamic septic phase, as measured by the reduction in the symptom score, hypothermia, and decrease in body weight. Overall mortality was reduced, but this reduction was mainly due to a decrease in the acute phase. This finding suggests that C5a might be an important mediator in the acute hyperdynamic septic response in this model. This conclusion is in agreement with the above mentioned clinical studies in which a possible relationship is suggested between C5a activation and septic shock. It is also in line with the reported attenuation of the acute response in experimental studies with cecal ligation and puncture and TNF-or endotoxin-induced sepsis. It has been suggested that C5a modulates the endotoxin-triggered TNF response. Endotoxin administration in C5-deficient mice resulted in markedly lower serum TNF-activity when compared with C5-sufficient mice (58). Since TNF production has been documented in the acute phase of our model, and pretreatment with anti-TNF antibodies could attenuate the acute response (59), the attenuation seen in C5-deficient mice could be TNF-mediated. However, late organ damage, as measured by the relative organ weights, was not reduced, suggesting that C5a might not be an important mediator in the late hypodynamic phase of this model. This finding is in agreement with the few clinical studies in which no relationship could be found between C5a activation and multiple organ dysfunction syndrome (5, 6). It is also in line with the studies done in the cecal ligation and puncture model where overall mortality did not decrease despite a decrease in acute mortality (37).

Since zymosan is also an important stimulator of macrophages (33), we hypothesize that activation of C5a is not the decisive factor associated with the development of multiple organ dysfunction syndrome in this model, and that other factors such as macrophage activation may be more important in this respect. Clinical indications, suggesting the importance of macrophage activation, are the association between neopterin concentrations and a poor outcome in sepsis and multiple trauma patients (60, 61). Neopterin is a stable inactive end product of macrophage metabolism and a marker of macrophage activity. A recent study (50) has shown that early after severe blunt trauma, neopterin/creatinine ratios were not significantly higher in patients developing multiple organ dysfunction syndrome. However, from 8 days after trauma, these ratios were significantly higher in those patients. This finding suggests that a late activation of macrophages is associated with the development of multiple organ dysfunction syndrome. In the TNF-endotoxin-model, C5-deficient mice were protected from TNF-endotoxin effects. However, when the TNF dose was increased, no protection occurred, suggesting that high doses of TNF act independent of the complement system (57). This finding shows that experimentally, other, more potent factors could be implicated in the pathogenesis of multiple organ dysfunction syndrome. Further support for this hypothesis comes from the fact that in our model, mortality could be reduced by elimination of liver and splenic macrophages with liposome encapsulated dichloro-methylene-diphosphonate before zymosan administration, suggesting that macrophage activation is also an important mediator (62).

We thus conclude that C5 deficiency attenuates the acute hyperdynamic response, but does not prevent organ damage in a model of zymosan-induced generalized inflammation. Thus, C5 is not the only factor involved in the late inflammatory response leading to multiple organ dysfunction syndrome.

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