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

Grard A. P. Nieuwenhuiizen, MD; M. P. Desirée Meyer, MD; Thijs Hendriks, PhD; R. Jan A. Goris, MD, PhD

**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.

**Subjects:** Twenty-five C5-deficient B2D10/Old and 25 C5-sufficient B2D10/New mice.

**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) x 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 C5α and C5b. C5α is both an anaphylatoxin and a chemotactic agent. The latter function is probably the most important since C5α is rapidly converted into C5α desarginine (des Arg), with concomitant loss of 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 C5α and C5α des Arg is specifically enhanced by Gc-globulin and is inactivated by chemotactic factor inactivator (16, 17). Pro-inflammatory cells possess a receptor for C5α (18). In addition to many inflammatory functions, C5α also has immunoregulatory activities. For example, C5α 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 cosogenic 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 (B10D2/0ld.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) x 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 fluid 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.

Organ damage of the surviving mice, assessed as the increase in relative organ weights of lungs, liver, spleen and kidneys is depicted in Figure 2. No significant differences were found between the two strains.

Bacteriology. All cultures of the peritoneal fluid of the surviving mice were sterile, thereby indicating the absence of bacterial peritonitis.

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
Critical Care Medicine

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 C5a/C5a des Arg ratio and terminal complement complex concentrations, but not of C5a concentrations (25, 41). Other studies (42, 43) could not confirm that the severity or mortality of sepsis is correlated with a persistent increase in C5a concentrations. On the other hand, a strong correlation was found between increased plasma concentrations of C5a and the development of ARDS (44), while C5a concentrations correlated with the severity of pulmonary failure (45). It also has been demonstrated that C5a is increased in bronchoalveolar lavage fluid in patients developing ARDS, with a normalization after ARDS had resolved (16). Chemotactic factor inactivator, which decreases C5a-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 C5a 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 C5a 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 C3a and C4a, but not of C5a, 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 proactivator and C4 concentrations, but not with C5a concentrations. Roumen et al. (50) demonstrated recently in patients with severe blunt trauma that an early increase in C3a and terminal complement complex concentrations early after severe blunt trauma were associated with the development of multiple organ dysfunction syndrome. However, only C3a/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 C5a since C5a, and to a lesser extent, C5a des Arg, are relatively unstable molecules and concentrations of C5a or C5a des Arg do not always reflect the level of C5a activation. C5a is rapidly cleared from the circulation and converted to C5a des Arg by serum carboxypeptidase. Furthermore, C5a 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 C5a 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 C5a. 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-C5a-antibodies resulted in a reduction of mortality, an attenuation of ARDS and decreases in the
systemic manifestations of sepsis. Peak C5a concentra-
tions were decreased and no significant differ-
ences were seen in C3a or C4a concentrations (55).
These experiments were confirmed by others in an 
endotoxic rat model (56) in which anti-C5a-antibod-
ies attenuated the hypotensive and vascular perme-
ability changes after endotoxin-induced shock. C5-
deficient B10D2/new mice showed less septic lung 
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, hemocoagulation and bowel injury. 
The latter study (57) also showed the importance of 
C5 since C5-deficient mice were not protected.

The present study shows that C5-deficient mice 
displayed an attenuated response in the acute hy-
perdynamic septic phase, as measured by the re-
duction in the symptom score, hypothermia, and 
decrease in body weight. Overall mortality was re-
duced, but this reduction was mainly due to a de-
crease 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 rela-
tionship 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 re-
sponse (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 punc-
ture 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 macro-
 phage activation may be more important in this 
respect. Clinical indications, suggesting the impor-
tance of macrophage activation, are the association 
between neopterin concentrations and a poor out-
come in sepsis and multiple trauma patients (60, 
61). Neopterin is a stable inactive end product of 
macrophage metabolism and a marker of macro-
 phage activity. A recent study (50) has shown that 
early after severe blunt trauma, neopterin/crea-
tinine 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 macro-
 phages is associated with the development of mul-
tiple 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 hy-
pothesis comes from the fact that in our model, 
mortality could be reduced by elimination of liver 
and splenic macrophages with liposome encapsu-
lated dichloro-methylene-diphosphonate before zy-
mosan 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 pre-
vent 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.

REFERENCES

1. Deitch EA: Multiple Organ Failure: Pathophysiology and 
2. Goria RJA, te Boekhorst TPA, Nuytinck JKS, et al: Multiple 
organ failure: Generalized autodestructive inflammation? 
Arch Surg 1985; 120:1109–1115 
3. Frank MM: Complement in the pathophysiology of human 
the complement system in trauma and infection. Surg Gyn
5. Heideman M, Hugli TE: Anaphylatoxin generation in mul-
complications and inflammatory mediators. Arch Surg 1986; 
121:886–890
47. Langlois PF, Gawryl MS: Accentuated formation of the terminal C5b-9 complement complex in patient plasma precedes development of the adult respiratory distress syndrome. Am Rev Respir Dis 1988; 138:368–375
49. Parsons PE, Giclas PC: The terminal complement complex (sC5b-9) is not specifically associated with the development of the adult respiratory distress syndrome. Am Rev Respir Dis 1988; 138:1272–1276
C5 DEFICIENCY IN A MODEL OF MULTIPLE ORGAN DYSFUNCTION SYNDROME

DIS 1990; 141:98–103


EDITORIAL APPROACH

Critical Care Medicine publishes original clinical investigations, outstanding laboratory studies, case reports of special importance, and manuscripts regarding technological advances in critical care medicine. In addition, review articles and articles of special concern to practitioners and scientists in the broad field of critical care are occasionally considered for publication. The publication of accompanying editorials is an important component of each issue of the journal. It is required that all manuscripts be original. Authors are expected to adhere strictly to the journal's Instructions for Authors, and authors assume full responsibility for the contents of their manuscripts. Critical Care Medicine is the official journal of the Society of Critical Care Medicine (SCCM). The Editors and Publisher of Critical Care Medicine and SCCM do not claim responsibility for statements or claims made by authors or advertisers, and publication of an article or advertisement within the journal is not an endorsement by the journal or SCCM of the conclusions of the authors or of the products manufactured by any advertiser.

Bart Chernow, MD, FCCM
Editor-in-Chief