Elimination of Various Subpopulations of Macrophages and the Development of Multiple-Organ Dysfunction Syndrome in Mice

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Objective: To evaluate the role of specific macrophage subpopulations in the development of zymosan-induced multiple-organ dysfunction syndrome by selective elimination of liver, splenic, alveolar, and peritoneal macrophages.

Design: Randomized animal trial.

Setting: Central animal laboratory at the University Hospital Nijmegen, Nijmegen, the Netherlands.

Animals: Male C57B1/6 mice.

Interventions: Elimination of macrophages was accomplished by administration of multilamellar liposomes that contained dichloromethylene bisphosphonate (C12MBP). Intravenous, intratracheal, and intraperitoneal administrations induced an elimination of liver and splenic, alveolar, and peritoneal and omental macrophages, respectively. Zymosan (1 mg/g) was injected intraperitoneally at day 0. The liposomes that contained C12MBP were administered before and after zymosan challenge. At day 12, all surviving mice were killed.

Main Outcome Measures: The body weights, temperatures, and mortality rates of the mice were monitored daily. Relative organ weights (ROWs) were calculated from the lungs, liver, spleen, and kidneys after the mice were killed.

Results: The liposomes that contained C12MBP, administered intravenously before or after zymosan challenge, did not induce significant changes in the body weight, temperature, or mortality rate. The ROW of the liver was significantly decreased in both treatment groups. Elimination of liver and splenic macrophages after zymosan challenge induced an increased ROW of the lung and a decreased ROW of the liver. The liposomes that contained C12MBP, administered intratracheally before zymosan challenge, completely prevented deaths. The body weights, temperatures, and ROWs of the mice were not changed. The liposomes that contained C12MBP, administered intraperitoneally, did not change the body weight, temperature, or ROW. The liposomes that contained C12MBP, administered intraperitoneally before zymosan challenge, increased the mortality from 50% to 90%.

Conclusions: These data show that the elimination of specific macrophage subpopulations and the elimination on specific time points in this model had differential effects, indicating a differential role of specific macrophage subpopulations, either protective or detrimental, in the development of multiple-organ dysfunction syndrome.

Arch Surg. 1997;132:533-539

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In normal host-defense mechanisms, an inflammatory response is teleologically beneficial and is meant to protect the host against injurious events. On the contrary, in pathological conditions (eg, the systemic inflammatory response syndrome [SIRS], the adult respiratory distress syndrome [ARDS], and the multiple-organ dysfunction syndrome [MODS]), an excessive generalized inflammatory response may have detrimental effects on the host.1,2 Macrophages play a pivotal role in normal host-defense mechanisms because of their antimicrobial phagocytic activity, antigen-presenting capacity, and regulation of the inflammatory response by their secretion of various immunoregulatory substances. However, evidence has been accumulating with regard to an excessive activation of macrophages that could induce a generalized dysregulated excessive inflammatory response, leading to the development of SIRS, ARDS, or MODS.4,5 This hypothesis is supported by various observations. First, administration of macrophage-derived cytokines to healthy volunteers produces a syndrome that closely resembles SIRS.7,8 Second, experimental and clinical data indicate a concurrent activation of macrophages during the development of SIRS, ARDS, and MODS, as demonstrated by increased levels of macrophage-derived products (eg, tumor necrosis factor [TNF], interleukin-1, and neopterin) with a positive correlation of elevated levels with the sub-
MATERIALS AND METHODS

ANIMALS

Inbred, specific pathogen-free, male C57Bl/6 mice (Charles Rivers, Wiga, Germany) (age range, 6-8 weeks) were adapted to handling in their cages for 7 days prior to the start of the actual experiment. Throughout the experiment, all mice had free access to water that was acidified with hydrochloric acid (to pH 3) and to standard mice laboratory chow (RMH-GS pellets [irradiated at 10 kGy], Hope Farms, Woerden, the Netherlands). The room temperature was kept constant at 21°C, and a 12-hour lighting cycle was maintained. The experiments were approved by the Animal Ethics Committee of the Medical Faculty of the University of Nijmegen, Nijmegen, the Netherlands.

ZYMOsan

Zymosan A (Sigma Chemical Co, St Louis, Mo), sterilized by use of γ-irradiation (5 kGy), was suspended (25 mg/mL) by high-frequency vibration in liquid paraffin (60-80 mPa, European Pharmacopoeia PA.5.68.81 CP846021). The suspension was sterilized by incubation in a water bath at 100°C for 80 minutes. One day before use, the suspension was heated to 40°C and dispersed by high-frequency vibration for 15 minutes. Sterility was confirmed by culture on a blood agar medium. The zymosan suspension was administered IP in a dose of 1 mg/g body weight.

PREPARATION OF CI2MBP-CONTAINING LIPOSOMES

Multilamellar liposomes were composed of phosphatidylycholine and cholesterol (molar ratio, 6:1) and contained CI2MBP dissolved in phosphate-buffered saline (PBS [0.25 mg/mL]). They were prepared as described earlier.22 In brief, multilamellar liposomes were composed of phosphatidylcholine and cholesterol (molar ratio, 6:1) and contained CI2MBP dissolved in phosphate-buffered saline (PBS [0.25 mg/mL]). They were prepared as described earlier.22 In brief,

RESULTS

Elimination of liver and splenic macrophages was confirmed by the absence of acid phosphatase activity of liver and splenic tissue (Figure 1) in the mice that were treated

ELIMINATION OF MACROPHAGE SUBPOPULATIONS

Elimination of Kupffer cells and splenic macrophages was achieved by an intravenous (IV) injection of 200 μL of liposomes that contained CI2MBP in the tail vein; this method has been demonstrated previously to achieve an elimination of these macrophages within 2 days.22 Alveolar macrophages were eliminated by administering CI2MBP-containing liposomes intratracheally (IT).27 The mice were fixed in an upright position under total anesthesia with 30 μL of a mixture (ratio, 4:3:7) of ketamine hydrochloride (100 mg/mL) (A.U.V., Cutijk, the Netherlands), xylazine hydrochloride (Rompun, Bayer, Leverkusen, Germany), and saline that was injected intramuscularly. Using a nylon tube (diameter, 0.46 mm) that was connected to a 1-mL syringe fixed in a micromanipulator, 100 μL of the CI2MBP-containing liposome suspension was administered through the glottis into the trachea. It has been demonstrated by this method that alveolar, but not interstitial, macrophages are eliminated within 3 to 5 hours.27 Elimination of peritoneal macrophages was accomplished by administering 100 μL of CI2MBP-containing liposomes IP, 2 times successively.28 The latter method has been demonstrated to eliminate peritoneal, omental, and, to a lesser extent, liver and splenic macrophages within 4 days.28

86 mg of phosphatidylcholine (Lipoid GmbH, Ludwigshafen, Germany) and 8 mg of cholesterol (Sigma Chemical Co) were dissolved in 10 mL of chloroform, and a lipid film was produced by low-vacuum rotary evaporation. Subsequently, 10 mL of the CI2MBP solution was added, and the suspension was left at room temperature for 2 hours, sonicated for 3 minutes at 20°C, and left for 2 hours at 37°C. The liposome suspension was then diluted in 100 mL of PBS, centrifuged at 100 000g for 30 minutes to remove free CI2MBP, and resuspended in 4 mL of PBS.
For each route of liposome administration, verification of macrophage elimination was performed on 3 animals, and the results were compared with those for untreated mice. Elimination of macrophages in the liver and spleen was verified by analyzing acid phosphatase activity in 8-μm cryostat tissue sections of liver and spleen 2 days after IV injection of Cl2MBP-containing liposomes. Acid phosphatase activity was demonstrated by incubation with 1% naphthol-AS-Bi—phosphate and 4% hexazotized pararosanilin for 30 minutes at 37°C. Elimination of alveolar macrophages was verified by quantification of macrophages in the alveolar lavage fluid at 2 days after IT administration of Cl2MBP-containing liposomes. After the lungs were dissected free, alveolar lavage was performed 3 times with 1 mL of PBS that contained 0.38% citrate. After staining with trypan blue, cells were counted using a hemocytometer chamber (Bürker counter, Tamson Co, Zoetermeer, the Netherlands). Cytospin preparations confirmed that more than 95% of the alveolar cells were macrophages. Elimination of the peritoneal macrophages was verified by quantification of macrophages in the peritoneal lavage fluid. The liposomes that contained Cl2MBP were administered IP at days −4 and −1. At day 0, peritoneal lavage was performed with 4 mL of PBS that contained 0.38% citrate. Quantification of cells was performed as described previously.

EXPERIMENTAL DESIGN

Three series of experiments were performed to assess the effects of macrophage elimination in the various tissues. Within each series, 3 groups of mice were used: a control group that received only zymosan and 2 experimental groups that received the Cl2MBP-containing liposome suspension either before or after administration of zymosan.

Thus, 3 groups of 15 animals were used to study the effects of IV liposome administration. The experimental groups received liposomes either 2 days before or 4 days after zymosan challenge. The effects of IT liposome administration were studied in 3 groups of 20 mice each. The experimental groups received liposomes either 3 days before or 4 days after zymosan challenge. Three groups of similar size were used to examine the effects of IP liposome administration, for which the experimental groups received liposomes either both 4 days and 1 day before or 1 day and 4 days after zymosan challenge.

In all mice, body weight and rectal temperature were measured daily. The clinical condition with possible symptoms such as loss of hemorrhagic fluid from the nose, conjunctiva, and mouth, lethargy, anorexia, hyperventilation, ruffled fur and loss of liquid stools, and mortality rate were monitored. On day 12, all surviving mice were bled and killed by cervical dislocation; the lungs with the trachea, kidneys, liver, and spleen were dissected free and weighed. Relative organ weights (ROWs) were calculated by the following formula: \[ \text{ROW} = \frac{\text{organ weight}}{\text{body weight}} \times 100\% \]

STATISTICAL ANALYSIS

Comparisons were only made between treatment groups and their specific control groups. Statistical analysis of biological parameters (body temperature and body weight) was performed using the distribution-free curve analysis according to Koziol et al.\(^{29}\) Since the zymosan-induced illness is characterized by distinct phases, comparisons were made separately for the course of the biological parameters in the acute phase (days 0-4) and late phase (days 8-12) of the model. The nonparametric Kruskal-Wallis and Wilcoxon 2-sample tests were used for statistical analysis of the ROWs. Noncontinuous data (mortality rates) were analyzed by use of the Fisher exact or \(\chi^2\) tests, when appropriate. Differences between groups were considered to be statistically significant at \(P<.05\).
Intravenous administration of liposomes after zymosan challenge led to significantly increased relative lung weights, while relative liver weights were significantly reduced in both groups that were tested with liposomes (Figure 3). No significant differences were noticed in relative spleen and kidney weights.

Intratracheal administration of liposomes that contained Cl2MBP before or after zymosan challenge did not result in significant differences in the course of the biological parameters (data not shown). While 25% of the animals died in the control group, no deaths occurred in the group that received liposomes before zymosan (P=.04), but elimination of alveolar macrophages after zymosan challenge did not significantly reduce the mortality rate (Figure 2). Organ damage, as measured by the ROWs, was not significantly changed by elimination of alveolar macrophages (Figure 3).

Intrapерitoneal administration of liposomes that contained Cl2MBP did not significantly change the course of body weight and temperature (data not shown). However, administration of Cl2MBP-containing liposomes before zymosan challenge induced a dramatic mortality rate. While mortality was approximately 50% in the control group and the group that received liposomes after zymosan, it reached 90% if animals were pretreated with liposomes (Figure 2; P=.02). The latter mortality was mainly due to the fact that the animals died in the early phase of this model. With only 2 animals surviving in this group, no reliable conclusions could be made with respect to organ damage. In the animals that were treated with liposomes after zymosan challenge, no statistically significant changes in the ROWs were observed (Figure 3).

**COMMENT**

This study shows that elimination of Kupffer cells and splenic macrophages (portal macrophages), either before or after zymosan challenge, is associated with lower, although not significantly, late deaths and significantly lower relative liver weights, indicating less liver damage. This is consistent with studies that have documented that excessive Kupffer cell activation is associated with alterations in liver function. Concomitantly, relative lung weights were higher when portal macrophages were eliminated after zymosan challenge. The latter observation may be explained in view of other studies that have hypothesized that ARDS and MODS could result from dysfunctional Kupffer cells, which spill over endotoxin to the lung and induce an excessive activation of alveolar macrophages; this phenomenon has been coined the "liver-lung axis." Indeed, alveolar macrophages are releasing much more TNF in response to lipopolysaccharide than Kupffer cells. Thus, by this mechanism, elimination of portal macrophages could lead to an activation of alveolar macrophages that results in increased lung damage.

Our data did not show adverse effects on the mortality rate by eliminating portal macrophages. This is in contrast with a study in the cecal ligation and puncture model, in which Kupffer cell blockade was achieved with gadolinium chloride. While increasing systemic immunity, this method of macrophage blockade increased the mortality rate. These discordant results could be explained because gadolinium chloride predominantly blocks phagocytic activity of Kupffer cells, leaving macrophage secretory activity relatively intact. The method, which we applied, physically eliminates almost all Kupffer cells, leaving no function intact. Furthermore, while the zymosan-induced generalized inflammation (ZIGI) model is largely independent of bacteria, the cecal ligation and puncture model is a slowly progressive bacteremic model, in which an intact host defense against bacteria is probably important. Apparently, the effects of manipulating macrophage function on the host defense can be detrimental in models or clinical situations that depend on the intact phagocytic activity of the host defense.

In this respect, our data of elimination of peritoneal macrophages are illustrative. Elimination of peritoneal macrophages before zymosan challenge induced a dramatic increase in the mortality rate, suggesting that in the early phase of this model, the function of peritoneal macrophages is essential for the host defense against intruding microorganisms or injurious agents like zymosan. However, elimination of peritoneal macrophages after zymosan challenge did not induce such effects since the mortality rate and ROWs were not significantly different. So, there appears to be a differential involvement of peritoneal macrophages in the development of MODS in this model: a protective role in the early phase and an indif-
ferent role in the late phase. In the ZIGI model, an early depression of peritoneal macrophage production capacity for TNF, interleukin-1, and interleukin-6 was observed during the first days after zymosan challenge, while they regained their capacity to produce cytokines in the late phase.\(^\text{21}\) Cecal ligation and puncture has also been shown to induce an early depression of peritoneal macrophage TNF production capacity and antigen presentation.\(^\text{25}\) Furthermore, these observations are interesting when comparing them with the observation of an increased susceptibility to a septic challenge after hemorrhage, since hemorrhage has been shown to decrease antigen-presenting and phagocytic activity while leaving the ex vivo secretory capacity of peritoneal macrophages relatively intact.\(^\text{3,7}\) Although it remains speculative, it could be that in the early phase, elimination of peritoneal macrophages before zymosan challenge enhances the effects of this early overall depression of peritoneal macrophage function, resulting in an increased mortality rate, comparable with the observed increased susceptibility for a septic challenge after hemorrhage. Furthermore, IP administration of \(\text{Cl}_{2}\text{MBP}\)-containing liposomes does not only eliminate IP and omental macrophages but also, to a lesser extent, liver and splenic macrophages.\(^\text{26}\) However, it appears unlikely that the observed effects that are seen after IP administration of \(\text{Cl}_{2}\text{MBP}\)-containing liposomes before zymosan challenge could be attributed to partial elimination of these macrophages, since elimination of Kupffer cells and splenic macrophages alone did not induce adverse effects. Hence, it seems that the observed high early mortality rate should be specifically attributed to the elimination of peritoneal macrophages.

Although not preventing organ damage or altering the course of the syndrome, it is striking that the eli-
nation of alveolar macrophages before zymosan challenge completely prevented mortality. This observation was consistently found in several pilot experiments (data not shown). This is in line with clinical data that have shown elevated levels of macrophage-derived cytokines (eg, TNF and interleukin-1) in bronchoalveolar lavage fluids in critically ill patients in whom ARDS develops. However, late elimination of alveolar macrophages did not alter the clinical course or mortality rate, nor did it influence organ damage. Thus, it appears that early alveolar activation is associated with mortality, while MODS appears to be independent of late alveolar macrophage activation. Other investigators, however, have shown experimentally that lung injury in the cecal ligation and puncture model was associated with a gradual increase in alveolar macrophage activation. Evidence for a less important role of the activated alveolar macrophage in the evolution of ARDS is supported by the clinical observation that early ARDS was associated with an increased number of macrophages, while a sustained elevated number of macrophages correlated with a decreased risk of death. These data suggest that early alveolar macrophage activation is associated with the onset of ARDS, while late alveolar macrophage activation is associated with a resolution of ARDS.

A major finding of this study was that selective elimination of any macrophage subpopulation, either before or after zymosan challenge, could not prevent both organ damage and death in this model. This could be explained by several hypotheses. First, during the development of MODS in this model, repopulation of some macrophages could already have been achieved in the late phase. Repopulation from the bone marrow requires 7 days for Kupffer cells and, depending on the type of macrophage, 7 days (red pulp macrophages) to 60 days (marginal zone macrophages) for splenic macrophages. Alveolar macrophage repopulation starts after 5 days and is complete after 18 days. Repopulation of peritoneal macrophages takes 7 days, and repopulation of macrophages of the omentum takes 7 to 28 days. When those macrophages were eliminated before zymosan challenge, their reappearance would be almost complete in the late phase, while it would be only partial when those macrophages were eliminated after the zymosan challenge. Hence, the reappearance of those macrophages in this phase could trigger the development of MODS if the agents or events that keep the macrophages activated are still present. Second, there are also indications in other models that the nondepleted macrophages demonstrate an increased activity. Thus, the elimination of a single population of macrophages could result in a compensatory activation of other macrophages or cell populations with an enhancement of the inflammatory response. In this respect, the observed increased lung organ damage after elimination of portal macrophages is an interesting phenomenon. Furthermore, elimination of peritoneal and alveolar macrophage populations was not complete; thus, the remaining macrophages could have hypothetically induced MODS in this model. On the other hand, the dramatic effects of intra-alveolar and IP liposomes before zymosan challenge suggest a significant contribution of the depletion of those macrophages to these phenomena.

The results could also be interpreted to support the notion that macrophage activation is not an important feature in the development of MODS. Indeed, the macrophage hypothesis has been questioned because of recent clinical data, indicating that preventive enhancement of macrophage activity by polyglutctriosyl glucopyranosyl glucan is beneficial for patients who are undergoing major surgery or multiple trauma. On the other hand, our data indicate that elimination of Kupffer cells and splenic macrophages attenuates liver damage. Furthermore, elimination of alveolar macrophages before zymosan challenge could fully prevent zymosan-induced death, indicating an important role for these macrophages in the development of MODS.

Our data further show that the elimination of certain macrophage subpopulations and the elimination on specific time points in this model has differential effects, indicating a differential role of specific macrophage subpopulations, either protective or detrimental, in the development of MODS. Peritoneal macrophages appear to be important in the host-defense mechanisms in the early phase, while contributing to the development of MODS in the late phase of this model. Kupffer cells and splenic macrophages appear to play an important pathophysiological role in all phases, while alveolar macrophages appear to be detrimental in the early phase. Thus, it remains to be determined which specific macrophage functions or populations are essential and need to be stimulated, and which are detrimental and need to be inhibited.

The Cl2MBP used in this study was provided by Boehringer Mannheim GmbH, Mannheim, Germany.

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REFERENCES

The MODS that progresses to multiple-organ failure is a serious problem for patients who undergo surgical procedures. Once multiple-organ failure develops, the mortality rate is high. Thus, information about the factors that are involved in producing this problem will eventually be beneficial for our patients. In this issue of the Archives, Nieuwenhuijzen and coworkers provide important information about macrophage activity in various organs (eg, the liver and spleen, the lungs [alveolar macrophages], and peritoneal cavity). Phagocytes in these organs or locations were destroyed by liposomes that contained a toxic agent. Then, the experimental animals were challenged by intravenous injection of liposome-encapsulated dichloromethylene diphosphonate: ultrastructural aspects of elimination of marginal zone macrophages. Virchows Arch. 1986;409:375-383.


