Objective: To investigate the alterations in circulating pro-inflammatory cytokines and cytokine production by peritoneal macrophages during the development of multiple organ dysfunction syndrome.

Design: Prospective, controlled laboratory study on zymosan-induced generalized inflammation in mice. Single intraperitoneal administration of zymosan induces, over a 12-day period, a triphasic illness in mice: the third phase, from day 6 onward, resembles multiple organ dysfunction syndrome.

Setting: Animal research laboratory.

Subjects: C57BL/6CRW mice received a single intraperitoneal dose of zymosan on day 0, and standard numbers of animals were killed at different time points up until day 12.

Measurements and Main Results: Plasma concentrations of interleukin (IL)-1α and IL-1β, IL-6, and tumor necrosis factor (TNF)-α were measured from 3 hrs to 12 days after administration of zymosan. At the same time points, both lipopolysaccharide-stimulated and unstimulated production of these cytokines by peritoneal macrophages were measured in vitro.

Plasma TNF and IL-6 concentrations transiently increased during the first 24 hrs after administration of zymosan. After 8 days, a prominent peak of biologically inactive TNF was observed. Both unstimulated and lipopolysaccharide-stimulated cytokine production by peritoneal cells showed profound changes during the experimental period.

Conclusions: These findings seem to confirm our hypothesis that the macrophages are in a continuously activated state and altered in their function, when the animals develop multiple organ dysfunction syndrome. Further studies are needed to elucidate what happens with these cytokines at the tissue level, to better understand the pathophysiology of multiple organ dysfunction syndrome. (Crit Care Med 1996; 24:1196–1202)

Key Words: multiple organ dysfunction syndrome; cytokines; tumor necrosis factor; interleukin-6; interleukin-1; animal model; mouse; peritoneal macrophage; zymosan

Of all patients now dying in the intensive care unit or in a surgical department, 50% to 60% die because of multiple organ dysfunction syndrome. Multiple organ dysfunction syndrome (1) is a cumulative sequence of progressive deterioration of function occurring in several organ systems, frequently seen after shock, in multiple trauma patients suffering from sepsis, severe burns wounds, or pancreatitis (2–7). This syndrome generally starts with lung failure and is followed by failure of the liver, gut, and kidneys, organs not necessarily directly involved in the primary disease. Furthermore, between the primary disease and the development of multiple organ dysfunction syndrome lies a period of days to weeks. Therefore, it is thought that endogenous and exogenous circulating factors mediate this multiple organ dysfunction syndrome (8, 9).

Macrophages are multifunctional cells that play a central role in host defense (10, 11). Not only do they process and present antigens to lymphocytes and exert antimicrobial activities, they also act as secretory cells during an inflammatory response, producing a variety of cytokines, such as interleukin (IL)-1, tumor necrosis factor (TNF)-α, and IL-6 (10, 12–15).

It is assumed that a continuously activated state of the macrophage can result in an abundant production of inflammatory mediators. Such an inflammatory response can occur systemically as a reaction to serious infections, and may contribute to the development of multiple organ dysfunction syndrome (16–19). It has been suggested that TNF exerts an important role in this respect (20, 21). However, the pathogenic pathways to multiple organ dysfunction syndrome remain to be elucidated. The patterns of cytokines like TNF, IL-1 and IL-6 in the circulation, as well as the capacity of macrophages to produce these substances during the development of multiple organ dysfunction syndrome, is not known because of variability in the clinical situation of multiple organ dysfunction syndrome patients (variety of underlying illness, medication), and because tissue macrophages are not easy to obtain in patients.

In our laboratory, an animal model for multiple organ dysfunction syndrome has been developed (9, 22–24). Rodents that receive intraperitoneal zymosan display zymosan-induced generalized inflammation and develop an illness, which ultimately leads to a clinical picture resembling multiple organ dysfunction syndrome. To gather insight into the possible role of various cytokines in its pathogenesis, we measured the patterns of circulating IL-1α and IL-1β, IL-6, and TNF-α in the subsequent phases of zymosan-
induced generalized inflammation in mice. In addition, we investigated the capacity of peritoneal macrophages, harvested in different phases of the model, to produce these cytokines.

MATERIALS AND METHODS

Animals. Experiments were performed using inbred C57BL/6 male mice (Charles River, WIGA, Germany), 6 to 8 wks old, weighing 20 to 25 g. The animals had free access to acidified water and standard chow (Hope Farms, Woerden, The Netherlands). The day/night cycle was 12/12. Before use, the animals were allowed to acclimatize for 1 wk. Three days before the actual experiment started, the animals were customized to daily handling to reduce stress during the experiment. The study protocol was approved by the Animal Ethics Review Committee of the Faculty of Medicine, University of Nijmegen.

Zymosan-Induced Generalized Inflammation. The animal model of zymosan-induced multiple organ dysfunction syndrome has been described previously (9, 22-24). Mice were given an aseptic intraperitoneal injection with zymosan (Sigma Chemical, St. Louis, MO) suspended at 25 mg/mL, in paraffin oil (60 to 80 milliPascal). The dose was 1 mg/g body weight. Zymosan A, a carbohydrate-rich cell wall preparation derived from the yeast Saccharomyces cerevisiae was sterilized by γ-irradiation (5 kiloGray). The particles were suspended in sterile paraffin oil, and high-frequency vibration during 1 hr was done to obtain a homogeneous suspension. After sonication, in batches of <6 mL, the suspension was sterilized again in a waterbath at 100°C for 80 mins. All suspensions were freshly made before use.

Body weight, body temperature, and behavior of the animals injected with the zymosan suspension were monitored for 12 days. At different time points after the zymosan injection, groups of animals (n = 14, unless otherwise indicated) were killed to obtain samples of blood, peritoneal cells, and tissues for analysis.

Collection of Plasma and Peritoneal Cells. Mice were anesthetized with ether and a blood sample was collected individually by means of eye extraction in capillary whole blood collectors. K$_2$-ethylenediaminetetraacetic acid-plasma was separated by centrifugation at 1500 g for 10 mins at 4°C, pooled (plasma of seven animals was pooled; at each time point. there were two pools available), aliquoted, and stored at -70°C until assayed. Prior experiments showed that pooling of plasma did not affect the outcome of cytokine measurements (unpublished information). After blood collection, the animals were killed by cervical dislocation, and 5 mL of ice-cold sterile phosphate buffered saline containing 0.38% citrate was injected into the peritoneal cavity. After gently massaging, the cell suspension was re-drawn under aseptic conditions. The cells were washed twice in cold phosphate buffered saline and resuspended in RPMI 1640 (Dutch modification), containing 0.1 M of sodium-pyruvate, 2 mM of L-glutamine (Gibco, Paisley, UK), and 0.05 mg/mL of gentamicin sulfate at a concentration of 1 x 10$^6$ viable cells/mL. Cell viability was determined by trypan blue exclusion.

Cells were plated into a 24-well tissue culture plate, 1 mL of suspension per well. Both unstimulated and stimulated (with lipopolysaccharide [10 µg/mL] from Escherichia coli, serotype 055:B5, Sigma Chemical Company) cytokine production was measured after a 24-hr incubation at 37°C (5% CO$_2$). Cytosin preparations of these cell suspensions were stained with Giemsa and May-Grunwald (Merck Diagnostica, Darmstadt, Germany).

Cytokine Assays. Plasma concentrations of the cytokines IL-1α, IL-1β, TNF-α, and IL-6, and the capacity of peritoneal macrophages to produce these cytokines were determined by assaying the plasma and supernatants of unstimulated and lipopolysaccharide-stimulated cultures. After incubation of peritoneal cells, the supernatants (for measuring IL-1β, TNF-α, and IL-6) and cells (for measuring cell associated IL-1α) were collected separately, aliquoted, and stored at -70°C until assay. Cells were frozen and thawed three times to release the cell-associated cytokine IL-1α. IL-1β, and TNF-α were measured by specific radioimmunoassay (RIA). Standards, culture supernatants, cell lysates, and plasma samples were diluted in an RIA-buffer (60 mM Na$_2$HPO$_4$,2H$_2$O; 12 mM EDTA; 1 mL sodium-azide [20%]; 2.5 g of bovine serum albumin [RIA-grade; Sigma Chemical]; 1 mL triton X-100; 25 mL of aprotonin [Bayer, Leverkusen, Germany] in 1 L of distilled water, pH of 7.4). One hundred microliters of an appropriate rabbit anticytokine-antiserum was added to 100 µL samples and standards and the tubes were kept on ice. After vortexing, the tubes were incubated for 24 hrs at 4°C. Subsequently, 100 µL of the appropriate 125I-labeled cytokine containing ~10,000 counts per minute were added to each tube, and incubation was continued for a further 24 hrs at 4°C. Then, 750 µL of RIA buffer containing 9% (weight/volume) polyethylene glycol 6000 (Merck, Darmstadt, Germany), and 3% (volume/volume) goat-antirabbit serum was added. The tubes were incubated for 20 mins at room temperature and then centrifugated at 1500 g for 15 mins. Supernatants were discarded carefully and quickly drained on absorbent paper. Remaining radioactivity was counted in a γ-counter. The radioactivity in control tubes (the nonspecific binding activity) was subtracted from samples and standards. Detection limits were between 0.05 and 0.10 ng/mL. IL-6 activity was determined, using the B9 hybridoma cell proliferation assay. B9 cells were cultured at a density of 5 x 10$^3$ cells/well in a microtiter plate, in 200 µL of Iscove's modified Dulbecco's Medium (IMDM) (Gibco, Paisley, UK) supplemented with 40 µg/mL gentamicin sulfate and 10% heat inactivated fetal calf serum, in the presence of either serial dilutions of culture supernatants or plasma. B9 cell proliferation was measured by [3H]-Thymidin incorporation (Amersham, UK) during the last 18 hrs of the 72-hr incubation. The standard curve obtained with human recombinant IL-6 was used to determine the bioactivity of IL-6 in the culture supernatants and plasma samples. One unit of IL-6 was the amount required to promote the half maximal growth of the B9 cells. When plasma samples were determined, 0.38% citrate was added to the culture medium to prevent clotting. This citrate concentration had no influence on the assay.

Statistical Analysis. The Wilcoxon test was used to examine differences between cytokine production by cells from control and experimental groups.
For such analyses, \( p < .05 \) was considered significant. The Mann-Whitney test was used to examine differences between the body weight, body temperature, and organ weights of control and experimental groups. When this test was used, \( p < .05 \) was considered significant.

**RESULTS**

Intraperitoneal injection with zymosan resulted in a three-phasic illness. During the first phase (days 0 to 2), the injection caused an acute peritonitis, rendering the animals very ill, as was apparent from weight loss, diarrhea, and lethargic behavior. Approximately 25% of the animals died during this phase. After 2 days, the animals seemed to recover, demonstrated by a gain in body weight and vitality. None of the animals died in this second phase (days 3 to 7). During the third phase (days 8 to 12), which started 7 to 8 days after administration of zymosan, the animals became ill again with weight loss, lethargic behavior, hypothermia, and dyspnea, suggesting the onset and development of multiple organ dysfunction syndrome. Autopsy of animals on day 12 showed hemorrhagic lungs, enlarged spleen and liver, and histopathologic changes, further indications for multiple organ dysfunction syndrome. Figure 1 shows the changes in body temperature and weight during the three phases of the model, illustrating the course of the illness. Further indications for organ damage or changes were the relative and absolute organ weights (Fig. 2). There was a significantly (\( p < .05 \), Fig. 2A) increased relative organ weight of liver, lung, and spleen. Also, the absolute organ weights (\( p < .05 \), Fig. 2B) of the lungs and spleen were significantly increased, while the absolute liver weight was not. Furthermore, there was a significant decrease in absolute kidney weight.

**Circulating Cytokines.** Within 3 hrs after injection with zymosan, increased circulating concentrations of TNF-\( \alpha \), as measured by RIA, were detected. These concentrations decreased to nondetectable values within 18 hrs (Fig. 3A). TNF-\( \alpha \) was not detectable again until 8 days after zymosan administration, when it started to increase again and remained increased until the animals were killed. Analysis of these samples with the L929 bioassay for TNF showed that the first peak of immunoreactive TNF-\( \alpha \) was also biologically active (5.8, 8.7, and 2.0 ng/mL at 3, 6, and 18 hrs after administration of zymosan, respectively), while the second immunoreactive TNF peak measured in the late phase was not (TNF was not detectable at 2, 5, 8, 10, 11, and 12 days after administration of zymosan). Figure 3B shows the course of IL-6 concentrations in plasma: very high concentrations were found from 3 to 18 hrs after zymosan injection. The concentrations decreased after 1 day, but remained ~10 times higher than the controls throughout the rest of the experiment; concentrations did not increase when TNF increased again. IL-1\( \alpha \) concentrations in plasma (Fig. 3C) were slightly above the values obtained in control animals (0.16 ng/mL vs. 0.09 ng/mL, respectively) 3 hrs after zymosan administration, and there was also a slight but transient increase at the beginning of the late phase (0.17 ng/mL at day 7). Circulating IL-1\( \beta \) was not detectable at any time.

**Cytokine Production By Peritoneal Cells.** Cytospin preparations at different phases in the model showed that >95% of the peritoneal cells were macrophages.

Directly after zymosan injection, unstimulated TNF-\( \alpha \) production (Fig. 1) was increased in control animals (open bars) and in animals 12 days after intraperitoneal administration of zymosan (solid bars). The relative organ weight is calculated as: wet organ weight/total body weight \( \times 100\% \). Data are expressed as mean \( \pm \) SEM (\( n = 11 \)). Denotes significant (\( p < .05 \)) difference between groups by Mann-Whitney test.
4) was significantly suppressed compared with production by cells derived from control animals. Within 8 hrs, the unstimulated production of TNF was back to control values. There was a significant, two-fold increase on day 3 (0.67 vs. 0.36 ng/mL in control, p < .05) in production of TNF compared with control. After that, the values remained significantly increased in this phase, except on day 5. From day 7 onward, the production decreased to control values, and at day 12 the TNF production was significantly decreased (0.17 vs. 0.36 ng/mL in control, p < .05).

Lipopolysaccharide-stimulated production of TNF-α was also suppressed initially but increased significantly within 18 hrs after administration of zymosan (1.56 vs. 0.93 ng/mL in control, p < .05; Fig. 4). Toward the end of the second phase, there was a four-fold increase in stimulated TNF production (4.09 ng/mL on day 6 vs. 0.93 ng/mL in control, p < .001). At the end of this phase and at the beginning of the third phase, there was a transient decrease. However, at all times the concentrations remained significantly increased above those concentrations measured in control animals (e.g., 1.27 ng/mL on day 9 vs. 0.93 ng/mL in the control group, p < .05). Thereafter, the production increased further.

The unstimulated production of IL-6 was significantly suppressed at all phases when compared with control values (p < .01 at all times, Fig. 4). Lipopolysaccharide-stimulated IL-6 production was significantly suppressed in the first phase, but in the second and third phase this production was similar to control values.

Figure 5 shows that unstimulated production of IL-1α was significantly suppressed (p < .001) throughout the experiment, except at 6, 7, and 8 days after administration of zymosan. Lipopolysaccharide-stimulated production of IL-1α was suppressed significantly at all time points in both the first and the second phase (p < .001 and p < .05, respectively). At the end of the second phase and in the third phase (from day 7 onward), the production levels were again similar to control.

Unstimulated production of IL-1β was transiently but significantly suppressed directly after administration of zymosan (Fig. 5). During the second phase (days 3 to 7) the production was also suppressed significantly (p < .01). However, it increased to control values at the beginning of the third phase. Lipopolysaccharide-stimulated production of IL-1β was significantly suppressed the first 6 hrs when compared with control values. Thereafter, it increased significantly (p < .05 at all time points from day 2 onward) and remained increased throughout the rest of the experiment.

**DISCUSSION**

Zymosan-induced generalized inflammation in the mouse clearly results in a triphasic illness. These three phases differ not only in terms of degree of illness but also in cytokine patterns.

In the first phase of the model, which reflects the shock state, circulating TNF peaks immediately after zymosan administration. This TNF peak is followed by an increase in circulating IL-6 concentrations. Production of TNF by peritoneal macrophages (stimulated as well as unstimulated) is significantly suppressed at this time, as is the production of IL-6. IL-1β is not detectable in the circulation, whereas IL-1α is detectable. The finding of detectable circulating IL-1α and nondetectable IL-1β seems to be different from the situation in humans, where IL-1β is the major cytokine
released and IL-1α is largely cell-associated. The absence of IL-1β in the circulation does not imply that there is no increase in production in the tissues. The fact that the production capacity for IL-1β by peritoneal cells is largely preserved during the first phase, could be taken as support for such a suggestion.

The cytokine patterns that we observe during the first phase partly agree with findings of Ayala and colleagues (25, 26), who found a marked spontaneous release of IL-1, IL-6, and TNF-α 1 hr after cecal ligation and puncture (CLP). Addition of endotoxin to peritoneal macrophage cultures from CLP mice had no further stimulatory effect. In the zymosan-induced generalized inflammation model, the unstimulated and stimulated IL-1α and IL-6 production is suppressed 3 hrs after zymosan administration. IL-1β and TNF-α production in our model is virtually unchanged during the first 24 hrs after zymosan administration. This result is in contrast with the findings of Ayala et al. (26), who found that only TNF-α remained unaltered 4 hrs and 24 hrs after CLP. In the CLP model, the blunting of lipopolysaccharide-inducible cytokine production probably represents the development of a kind of tolerance phenomenon as is also seen during sepsis (27). Our study shows a more disparate cytokine response.

During the second phase, there is no TNF-α detectable in the circulation, while IL-6 concentrations are supranormal. Stimulated and unstimulated production of TNF-α is significantly increased when compared with production of cells collected from control animals during this phase. The conditions of the macrophages that produce these cytokines in vitro are not necessarily the same as in vivo, but the in vitro conditions represent at least two extreme situations (with or without triggering agent) that could occur within the peritoneal cavity. The total number of cells in the peritoneal cavity 2 days after zymosan administration is ten times higher than in control animals. This increase in cell number means that there is an enormous production capacity for TNF-α in the peritoneal cavity. Stein and Gordon (28) found that zymosan administration results in a prolonged release of TNF, suggesting that the undegraded intracellular zymosan particles cause ongoing release of TNF. Such phenomenon could also happen in vivo, when zymosan is present, although there is no TNF-α detectable in the circulation. Apparently, the animals can handle the excessive TNF-α production capacity during this phase.

IL-6 production by peritoneal cells in this phase is significantly decreased (unstimulated) or similar to normal (stimulated). Considering again the ten-fold increase in cells in the peritoneal cavity, it seems possible that the slightly increased concentrations found in the circulation merely reflect this increased number of cells.

Circulating IL-1α concentrations are increased at the end of the second phase, while unstimulated as well as stimulated production by peritoneal cells is normal at the end of this phase. As for IL-6, the increased cell number could be responsible for the increase found in the circulation.

Unstimulated production of IL-1β is significantly reduced. However, considering the cell number in the peritoneal cavity, the total production capacity could be much higher than in controls. Stimulated IL-1β production is significantly increased. However, circulating concentrations are neither found in these animals, nor in control animals. As in the first phase, the activity of IL-1β seems to be confined to the local inflammatory process. The suggestion that the cells develop a kind of tolerance (25) is also not true in this phase of the model.

During the most interesting phase, the third phase, the animals develop the multiple organ dysfunction syndrome. Immunoreactive TNF-α concentrations in the circulation increase again, while the unstimulated production by peritoneal cells decreases below control values and stimulated production increases significantly above control.

IL-6 does not increase to high concentrations again in the circulation.
The present study shows a complex pattern of circulating cytokines and production of these cytokines by peritoneal macrophages during the subsequent phases after zymosan administration.

There are various possibilities regarding the contribution of peritoneal cells to circulating cytokine concentrations. It is possible that the cells are in a continuously activated state, due to the persisting zymosan stimulation. The fact that unstimulated production of all four cytokines is similar to normal or even below normal during the third phase does not necessarily contradict the role of zymosan as a continuous stimulus, because the production is measured in vitro under circumstances different from those present in vivo (29). This fact makes it difficult to predict what is actually happening in the peritoneal cavity during different phases in the zymosan-induced generalized inflammation model.

The present study shows a complex pattern of circulating cytokines and production of these cytokines by peritoneal macrophages during the subsequent phases after zymosan administration. The peritoneal macrophage is altered in its capacity to produce cytokines after being in contact with zymosan when compared with the normal situation. There seem to be differential changes in stimulated as well as unstimulated production of the four cytokines studied here, but it seems a differentiated continuous response. Furthermore, the changes in circulating cytokines differ in each phase of the model. The contribution of other macrophage populations to these changes needs to be elucidated. In the third phase, when there are foci of inflammation in liver and lungs, the macrophage populations in these tissues are likely to be altered in their function as well. If these macrophages contribute to the circulating concentrations and to remote tissue damage. Considering that numbers of cells in the peritoneal cavity are similar to control animals during this phase, other populations of cells probably contribute to the slightly increased concentrations in the circulation. Further studies are being conducted to elucidate this issue.

Circulating IL-1β concentrations are not detectable, while stimulated production is significantly increased and unstimulated production by peritoneal cells is virtually normal. Apparently, IL-1β is restricted to the cellular level or scavenged by other proteins.

Circulating IL-1α concentrations are similar to control values, whereas unstimulated production is significantly suppressed and stimulated production is similar to control values. These data could imply that the IL-1α found in the circulation is generated at a site other than the peritoneal cavity.

but remains at a slightly increased concentration, whereas unstimulated production stays significantly reduced when compared with control values. The TNF-α peak in this phase is not followed by an IL-6 peak as is usually seen. However, it appears that the immunoreactive TNF-α found in the circulation at this time was largely biologically inactive, possibly because of the presence of circulating soluble receptors.

There is considerable lung damage that can be seen macroscopically (hemorrhages, edema) and which is apparent from the breathing difficulties of the animals. Also, there are foci of inflammation in the liver. Cytokines such as TNF, acting at the tissue level, are responsible for these abnormalities and thus contribute to the onset and development of multiple organ dysfunction syndrome, as suggested by others (20, 21) on the basis of data from patient studies.

We do not know to what extent cytokines produced by peritoneal cells contribute to the circulating concentrations and to remote tissue damage. Considering that numbers of cells in the peritoneal cavity are similar to control animals during this phase, other populations of cells probably contribute to the slightly increased concentrations in the circulation. Further studies are being conducted to elucidate this issue.

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are triggered to produce cytokines, they too could contribute to the spillover found in the circulation. The TNF-α peak found in this phase is biologically inactive, but the supranormal biologically active IL-6 concentrations are not inactivated or cleared. This inactivation and differential clearing may possibly contribute to, or be a result of, insufficient regulation of the immune response and may thus lead to multiple organ dysfunction syndrome.

These findings seem to confirm our hypothesis that the macrophages are in a continuously activated state, and altered in their function, when the animals develop multiple organ dysfunction syndrome. Further studies are needed to elucidate what happens with these cytokines at the tissue level, to better understand the pathophysiology of multiple organ dysfunction syndrome.

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REFERENCES