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Increasing Cytotoxic Activity and Production of Reactive Oxygen and Nitrogen Intermediates by Peritoneal Macrophages During the Development of Multiple Organ Dysfunction Syndrome in Mice

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A major problem in the intensive care unit nowadays is the development of multiple organ dysfunction syndrome (MODS), a cumulative sequence of progressive deterioration of organ functions. While the pathogenic pathways of MODS remain to be elucidated, it is assumed that cells of the host defence system, especially the macrophages, are altered in their function. During the development of MODS it is assumed that macrophages are overactivated and that an exaggerated inflammatory response may contribute to its pathogenesis. In order to gain insight into the alterations of the functional status of the macrophage during the development of MODS, a series of macrophage functions was measured in the subsequent phases of zymosan induced generalized inflammation in mice. Male C57BL/6 mice received a single dose of zymosan intraperitoneally and groups of animals were killed after 2, 5, 8, and 12 days. Peritoneal macrophages were collected for *in vitro* assessment of the ADCC, the production of superoxide (O_2^-) and nitric oxide (NO), and complement mediated phagocytosis and intracellular killing of *Staphylococcus aureus*. A single intraperitoneal injection with zymosan resulted in a three-phase illness. During the third phase the animals developed MODS-like symptoms. Peritoneal cells from control animals produced very low to non-detectable amounts of O_2^- and NO, and the cytotoxic activity was also low. During the development of MODS, from day 7 onwards, the ability to produce O_2^- and NO_2^- became strongly elevated, as did the cytotoxic activity. These findings are in parallel with the development of MODS whereas the phagocytic and killing capacity remained essentially unaltered. The changes found could be detrimental for the organism, thus possibly contributing to the onset and development of MODS.

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INTRODUCTION

A major problem in the intensive care unit nowadays is the development of the multiple organ dysfunction syndrome (MODS) by patients suffering from multiple trauma, shock, severe burn wounds, or pancreatitis [1–3]. It is a cumulative sequence of progressive deterioration of function occurring in

several organ systems, starting mostly with lung failure and followed by failure of the liver, gut, and kidneys, organs which are not necessarily directly involved in the primary disease [1]. While the pathogenic pathways of MODS remain to be elucidated, it is assumed that cells of the host defence system, especially macrophages, are altered in their functions. Macrophages occupy a central position in host defence.

Not only do they synthesize and release many soluble bioactive substances [4, 5] into their micro-environment or the bloodstream, they also possess cytotoxic and microbicidal activities [6, 7] as important host defence mechanisms against a variety of invading micro-organisms and viruses.

The production of reactive oxygen intermediates such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are important factors contributing to the defence. O_2^- is one of the immediate products of the respiratory burst, and under normal conditions it is used for the synthesis of microbicidal oxidants such as oxidized halogens and oxidizing radicals [6, 8].

In addition, the L-arginine dependent generation of reactive nitrogen intermediates is identified as a nonspecific microbicidal mechanism of the activated mouse macrophage. Nitric oxide (NO) is a highly soluble free radical with diverse roles, including regulation of vascular resistance, blood pressure, and signal transduction in a variety of tissues [9, 10]. Both radicals, when produced in excess, can cause severe tissue damage either directly or indirectly [11].

In our laboratory, we have developed an animal model for MODS [1, 12–14]. Rodents which receive a single dose of intraperitoneal zymosan, which is a potent activator of the complement system, display generalized inflammation and develop an illness which ultimately leads to a clinical picture resembling MODS. Our hypothesis is that during the development of MODS macrophages are overactivated and that an exaggerated inflammatory response may contribute to its pathogenesis [15–18]. Therefore, we examined whether macrophage phagocytic and cytotoxic activity, and the production of superoxide and nitric oxide were altered when MODS is induced in this model and thus possibly contribute to the organ damage.

MATERIALS AND METHODS

Animals. Experiments were performed using inbred C57BL/6 male mice, 6–8 weeks old, weighing 20–25 g (Charles River, WIGA, Germany). The animals were fed standard chow (Hope Farms RMB-H, Woerden, The Netherlands) and acidified water *ad libitum*. The day/night cycle was 12/12. Before use, the animals were allowed to acclimatize for 1 week. Three days before administration of zymosan the body weight and temperature were measured daily in order to reduce stress during the experiment.

The experiments were approved by the Animal Ethics Review Committee of the Faculty of Medicine, University of Nijmegen.

Materials. Zymosan A, ferricytochrome C, phorbol myristate-acetate (PMA), superoxide dismutase (SOD), lipopolysaccharide (LPS) from *E. Coli* serotype 055:B5, bovine serum albumin were all purchased from Sigma (St. Louis, MO, USA). Sulfanilamide, naphthylethylene diamine dihydrochloride and H_3PO_4 for preparation of the Griess reagent was also purchased from Sigma.

Culture medium for determination of superoxide and NO production consisted of RPMI-1640, 2 mM L-glutamine, and 40 mg/l gentamycin sulfate and were all purchased from Gibco, Paisley, UK. For measuring the cytotoxicity 10% heat-inactivated fetal calf

serum (FCS, Gibco) was added. Sodium-chromate (^{51}Cr) was purchased from the Radiochemical Centre, Amersham, UK.

Hank's balanced salt solution (HBSS, Gibco) was supplemented with 10 mM Hepes (Sigma) and 0.1% gelatin (Difco) for *in vitro* assessment of phagocytosis and intracellular killing (HBSS+). *Staphylococcus aureus* type 42D was a serum resistant strain kindly provided by Dr J. Curfs, Department of Medical Microbiology, Academic Hospital Nijmegen, the Netherlands. Diagnostic sensitivity medium agar (DST) plates and nutrient broth (both from Oxoid) were used for culturing the micro-organisms.

Zymosan-induced generalized inflammation. This animal model of the multiple organ dysfunction syndrome has been described extensively [15–19]. In short, mice were given an aseptic intraperitoneal injection with zymosan suspended at 25 mg/ml, in paraffin oil (60–80 mPa). 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 gamma-radiation (5 kGy). The particles were suspended in sterile paraffin oil by high frequency vibration for 1 h. After sonication, the suspension was sterilized once more in a waterbath at 100°C for 80 min. All suspensions were freshly made before use.

Body weight, body temperature, and behaviour of the animals injected with the zymosan suspension were monitored for 12 days. At different time points after zymosan injection groups of animals were killed to obtain peritoneal cells for further experiments.

Collection of peritoneal cells. After cervical dislocation 5 ml of ice-cold sterile phosphate buffered saline (PBS) containing 0.38% citrate was injected into the peritoneum. The PBS was withdrawn under aseptic conditions, cells were washed twice in cold PBS, and resuspended in the different media used in the experiments as described below. Unless otherwise indicated, cells were pooled and viability was determined by trypan blue exclusion. Purity of the macrophage population was > 95% at all time points, as was determined on cytopspin preparations by Giemsa and May-Grunwald staining (Merck Diagnostica, Darmstadt, Germany).

Determination of superoxide production. The production of superoxide (O_2^-) by macrophages was measured by the reduction of ferricytochrome C using the method described by Pick and Mizel [20]. Peritoneal cells (1×10^6 cells/ml, 200 μ l/well) were incubated in flat-bottomed microtitre plates in culture medium. After allowing the cells to adhere for 2.5 h the supernatants were removed and the cells were incubated with reaction solution containing 160 μ M ferricytochrome C and, if indicated, with 100 ng/ml PMA in HBSS. The amount of ferricytochrome C reduced by O_2^- was measured as the change in absorbance at 545 nm with an ELISA microplate reader. Wells containing medium and ferricytochrome C only were used as controls. The amount of O_2^- production per well was calculated as reported by Pick and Mizel using the formula:

$$\text{nmoles } O_2^- \text{ per well} = (A_{545}) \times 100/6.3$$

Preliminary experiments showed that O_2^- production by peritoneal cells was completely inhibited by the presence of SOD. In order to correct for loss of non-adherent cells, a duplicate plate was treated the same as the plate for the determination of superoxide up to the point when the cytochrome C was added. Instead of the reaction mixture, HBSS and 100 μ l of Isoton (containing 10% Zap-o-globin) was added and cells were counted on a Coulter Counter ($T_1 = 3.5 \mu$ m).

Determination of nitrite production as a measure for NO. The nitrite (NO_2^-) concentration is an indirect measure of NO synthesis.

peritoneal cells were resuspended in RPMI-1640 medium at a concentration of 1×10^6 viable cells/ml, and 1 ml was plated into a 24-well tissue culture plate. In order to measure both stimulated and unstimulated NO_2^- generation cells were incubated for 24 h at 37°C (5% CO_2) with or without addition of $10 \mu\text{g/ml}$ LPS, respectively. $100 \mu\text{l}$ aliquots from these cultures were incubated with an equal volume of Griess reagent (4 mM naphthylene-diamine-dihydrochloride, 0.26 M H_3PO_4 , and 58 mM sulfanilamide in distilled water, stored in the dark at room temperature until use for 10 min at room temperature. The absorbance at 540 nm was determined in a microtitre plate reader. Nitrite was quantified using NaNO_2 as a standard. Standards were measured in triplicate, samples from individual cultures from 8 to 14 mice per time point were measured in duplicate.

In vitro assessment of antibody dependent cell-mediated cytotoxicity (ADCC). The ADCC capacity of peritoneal cells was studied using a ^{51}Cr -release assay, using antibody-coated human red blood cells (HRBC) as target cells, as described by van de Winkel *et al.* [21]. Briefly, equal volumes of effector cells (peritoneal cells from mice at different time points after administration of zymosan) and ^{51}Cr labelled sensitized HRBC (sensitizing antibody: mouse-anti-human-antiglycophorin, IgG2a, a kind gift from L. Aarden, CLB, Amsterdam) were mixed in microtitre wells. After incubation for 18 h at 37°C , half the supernatant was removed for estimation of ^{51}Cr release in a LKB gamma counter. All tests were carried out in triplicate. The results were calculated as follows:

$$\% \text{lysis} = \frac{(\text{cpm}_{\text{sample}} - \text{cpm}_{\text{spontaneous}})}{(\text{cpm}_{\text{max}} - \text{cpm}_{\text{spontaneous}})} \times 100\%$$

$\text{cpm}_{\text{sample}}$ = mean cpm of test sample; $\text{cpm}_{\text{spontaneous}}$ = mean cpm of spontaneous ^{51}Cr release (i.e. release of ^{51}Cr by labelled target cells in medium only); and cpm_{max} = mean cpm of the maximal ^{51}Cr release, obtained by addition of 20% Zap-o-globin (Coulter) to the target cells.

In vitro assessment of phagocytosis and intracellular killing. The *in vitro* assessment of phagocytosis and intracellular killing was carried out according to the methods developed by Leijh *et al.* [22] with minor adjustments. Briefly, phagocytosis was measured by incubating peritoneal cells with live non-opsonized *S. aureus* at a ratio of 1:1 in HBSS+ in the presence of 10% normal mouse serum (NMS) under continuous rotation at 37°C . At specified time points $100 \mu\text{l}$ of the suspension was added to ice-cold HBSS+ to stop the phagocytic action. After centrifugation, a 10-fold serial dilution in saline was made of $100 \mu\text{l}$ of the supernatant, which contained only non-ingested bacteria. Aliquots of $100 \mu\text{l}$ were pipetted on DST plates, incubated for 18 h at 37°C and subsequently the colonies were counted. The number of viable bacteria in suspension at each time point was calculated from the means of duplicate plates. To correct for growth of the micro-organisms during the experiment the same number of micro-organisms were incubated in HBSS+ without peritoneal cells and treated likewise.

Pre-opsonization of *S. aureus* for intracellular killing measurements was carried out by incubation with 10% NMS for 30 min at 37°C . The suspension was then washed and resuspended at an appropriate concentration and kept on ice until use.

The rate of intracellular killing was measured independently from the rate of phagocytosis by allowing peritoneal cells to ingest pre-opsonized micro-organisms during incubation at 37°C for 20 min under continuous rotation. Phagocytosis was terminated by placing the tubes on ice. The cells were washed thoroughly to remove all the

extracellular micro-organisms and resuspended in the same volume of HBSS+. Intracellular killing started after adding 10% NMS and incubation at 37°C . At specified time points $100 \mu\text{l}$ sample of the suspension was removed and added to ice-cold distilled water containing 0.1% BSA. The peritoneal cells were lysed by mixing the suspension vigorously. The number of viable intracellular micro-organisms was determined in the same way as described above for the *in vitro* assessment of phagocytosis.

Statistical analysis. Statistical methods are mentioned with the results.

RESULTS

Zymosan-induced generalized inflammation

Intraperitoneal injection with zymosan resulted in a three-phase illness. Figure 1A & B shows the changes in body temperature and body weight, respectively, during the three phases of the model, illustrating the course of the illness. During the first phase (days 0–2), the injection caused an acute peritonitis, rendering the animals very ill, as was apparent from weight loss (Fig. 1A), diarrhoea, and lethargic behaviour. Approximately 25% of the animals died during this phase. After 2 days, the animals seemed to recover. None of the animals died in this second phase (days 3–6). During the third phase (days 7–12), the animals became ill again with weight loss, lethargic behaviour, hypothermia, and dyspnoea, suggesting the onset and development of MODS. Autopsy of animals at day 12 showed haemorrhagic lungs, enlarged spleen and liver, and histopathologic changes in these organs. Further parameters for organ damage/changes were the absolute and relative organ weights (Fig. 2 A & B, respectively). There was a significant ($P < 0.05$) increase in relative organ weight of liver, lung, and spleen. Also, the absolute organ weight of the lungs and spleen was increased significantly ($P < 0.05$).

Superoxide production by peritoneal cells

Peritoneal cells derived from control animals produced relatively low amounts of superoxide (Fig. 3: $1.8 \text{ nmoles}/1 \times 10^6$ cells). Two days after administration of zymosan the production of superoxide was slightly elevated compared to control. This rise in production continued 5 days after administration. Eight and 12 days after administration of zymosan the production of superoxide was significantly higher ($P < 0.05$) than production by cells derived from control animals ($36 \text{ nmoles}/1 \times 10^6$ cells and $45 \text{ nmoles}/1 \times 10^6$ cells, respectively).

Nitric oxide production by peritoneal cells

Unstimulated peritoneal cells from control animals produced non-detectable amounts of nitrite. This production was not significantly elevated until 1 day after administration of zymosan when it increased significantly compared to control values, and remained significantly elevated in all the following phases of the model ($P < 0.05$, Fig. 4A), except at day 5.

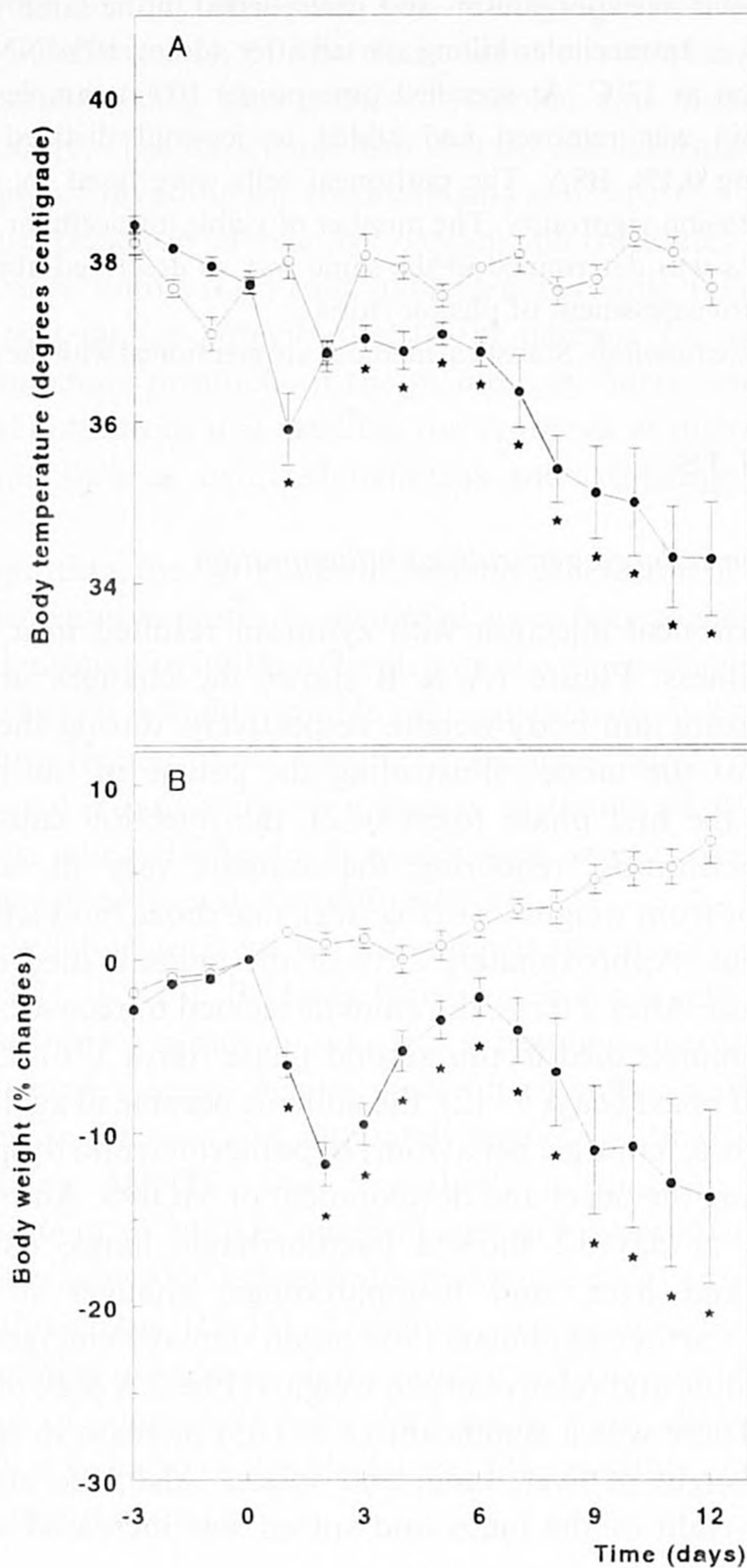


Fig. 1. Course of body temperature and body weight. Body temperature (A) and changes in body weight (B) in a control group (○; *n* = 8) and a group which received intraperitoneal zymosan at day zero (●; *n* = 8). Data represent mean values and SEM; *significant difference (*P* < 0.05 by Mann-Whitney test) between groups.

The LPS-stimulated production of nitrite was significantly inhibited until 1 day after administration of zymosan (Fig. 4B). At that time point the production was comparable to that of cells derived from control animals, but at day 2 it decreased significantly. However, after that (day 3) the stimulated production increased significantly above control values and remained significantly elevated throughout the rest of the experiment.

Antibody dependent cellular cytotoxicity

Figure 5 represents the ADCC capacity of peritoneal cells at different time points of the model. Two and 5 days after administration of zymosan the cytotoxic capacity of

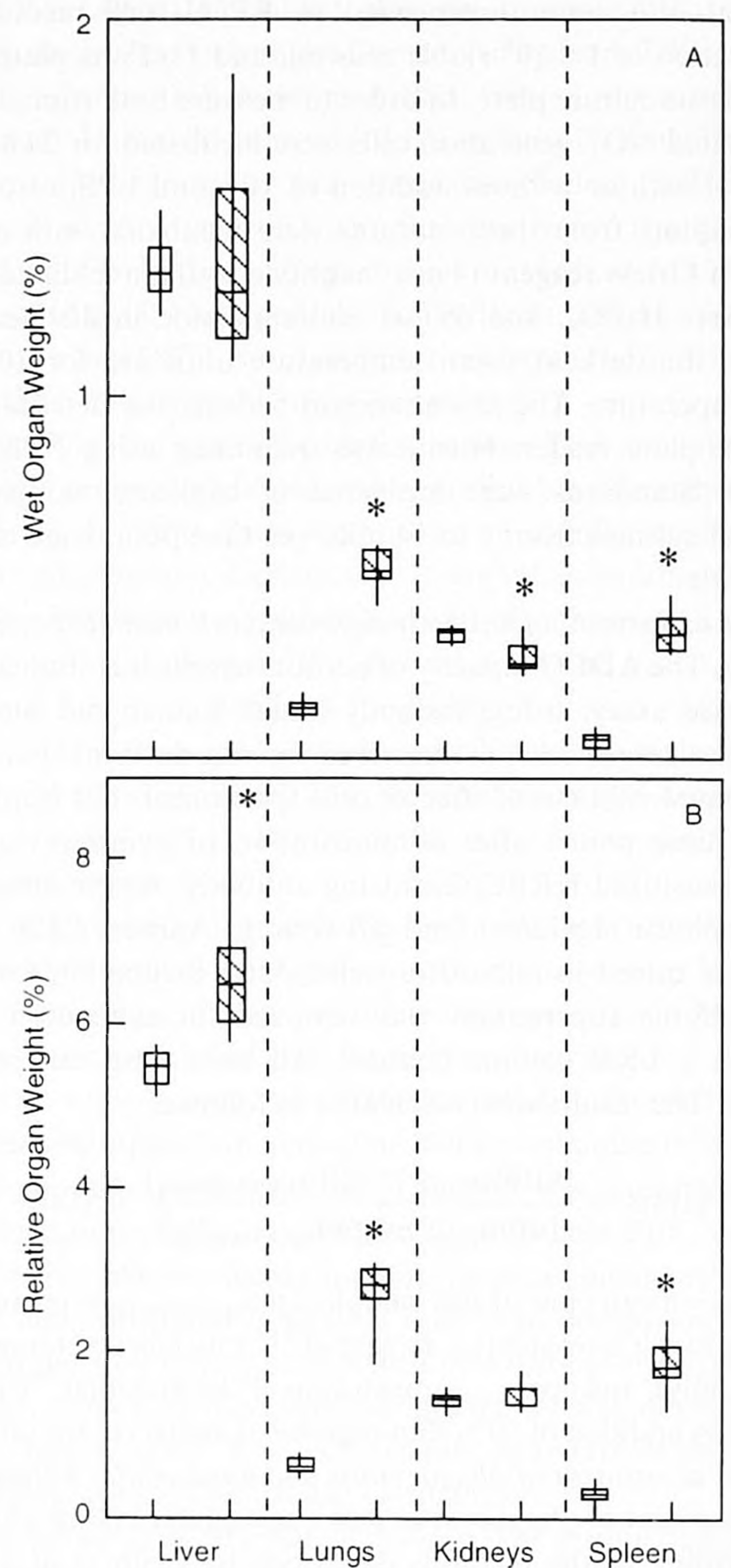


Fig. 2. Organ weight. Both absolute (A) and relative (B) organ weight in control animals (open bars, *n* = 8) and in animals 12 days after administration of zymosan (hatched bars, *n* = 8). The relative organ weight is calculated as: wet organ weight/total body weight × 100%. Data are expressed as medians in box plots ranging from the 25th to 75th percentile. Bars indicate 5th and 95th percentile. *Significant difference (*P* < 0.05 by Kruskal-Wallis test) between groups.

peritoneal macrophages at effector/target ratio 3:1 was decreased when compared to control. Eight days after administration of zymosan, lysis first occurred at a lower effector/target ratio (shift to the left), and when the effector/target ratio increased, the percentage lysis was higher (shift upwards), when compared to control. Twelve days after administration of zymosan the percentage lysis was even higher.

Phagocytosis and intracellular killing

Within 10 min the peritoneal cells from untreated mice (control in Fig. 6A) ingested 35% of the micro-organisms, which

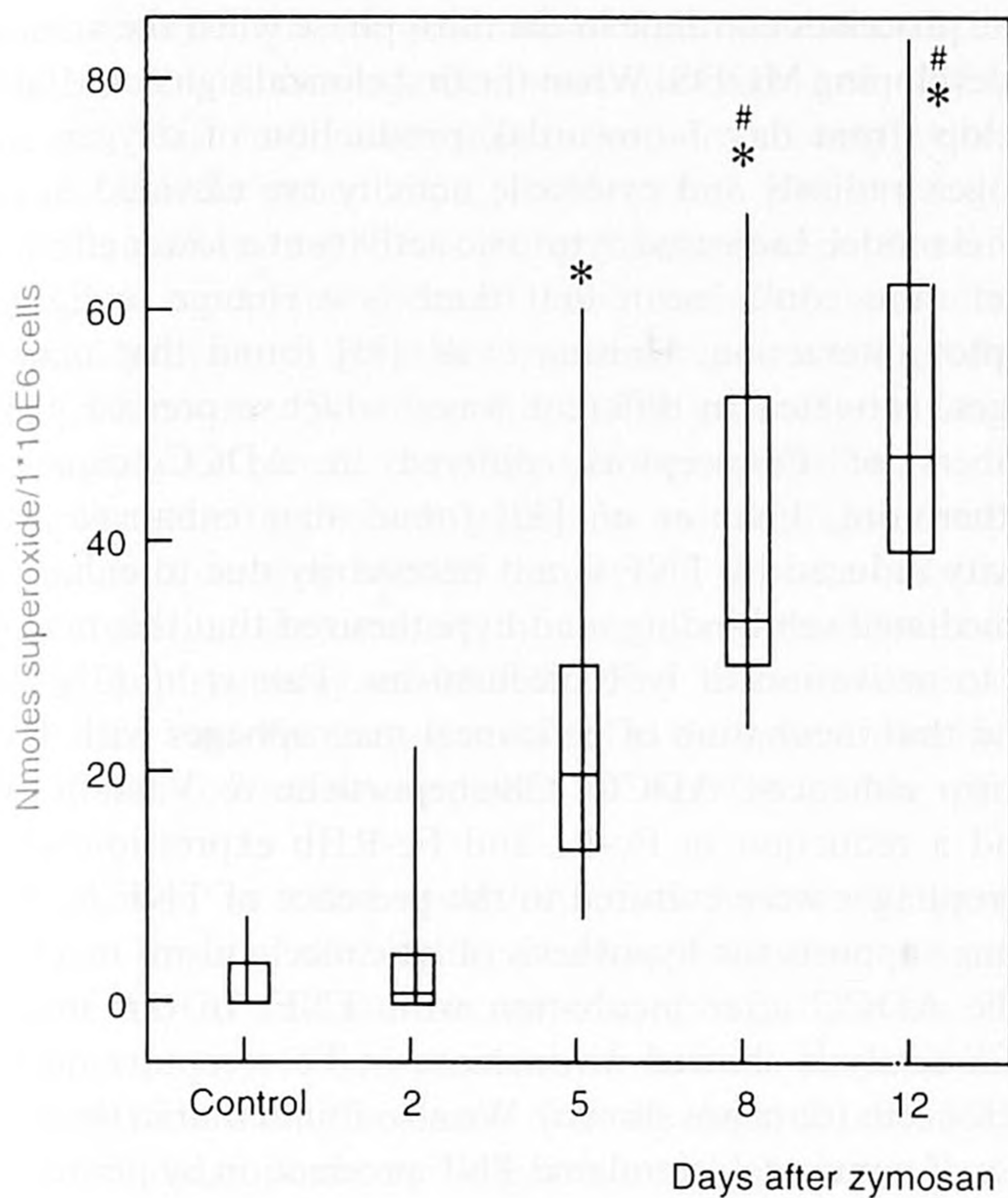


Fig. 3. Superoxide production by peritoneal cells. PMA-stimulated superoxide production by peritoneal cells derived from mice in different phases of the model. Data are from observations on individual cultures from 8 to 14 mice and are expressed as medians in box plots ranging from the 25th to 75th percentile. Bars indicate 5th and 95th percentile. *Significant difference ($P < 0.05$ by Kruskal-Wallis test) between control and experimental groups; #significant difference between the day 2 group and experimental groups.

increased to a maximum of 45% after 20 min of incubation. Eight days after administration of zymosan peritoneal macrophages showed a decrease in the rate of phagocytosis within the first 10 min when compared to the control value. Furthermore, at all time points in the model, the percentage of micro-organisms ingested after 30 min of incubation was similar to findings in untreated mice.

Peritoneal macrophages of untreated mice were able to kill more than 50% of the micro-organisms that were present intracellularly within 30 min after the start of the incubation (control in Fig. 6B). This capacity decreased 2 days after administration of zymosan, but was comparable to control again at the latter stages of the experiment.

DISCUSSION

In this study we have found that certain macrophage functions change during the development of MODS (production of oxygen and nitrogen radicals, ADCC), whereas others (phagocytosis and intracellular killing) remain virtually unaffected. The development of MODS in the zymosan-induced generalized inflammation model is preceded by two clinically distinct phases: the first phase in which the animals develop

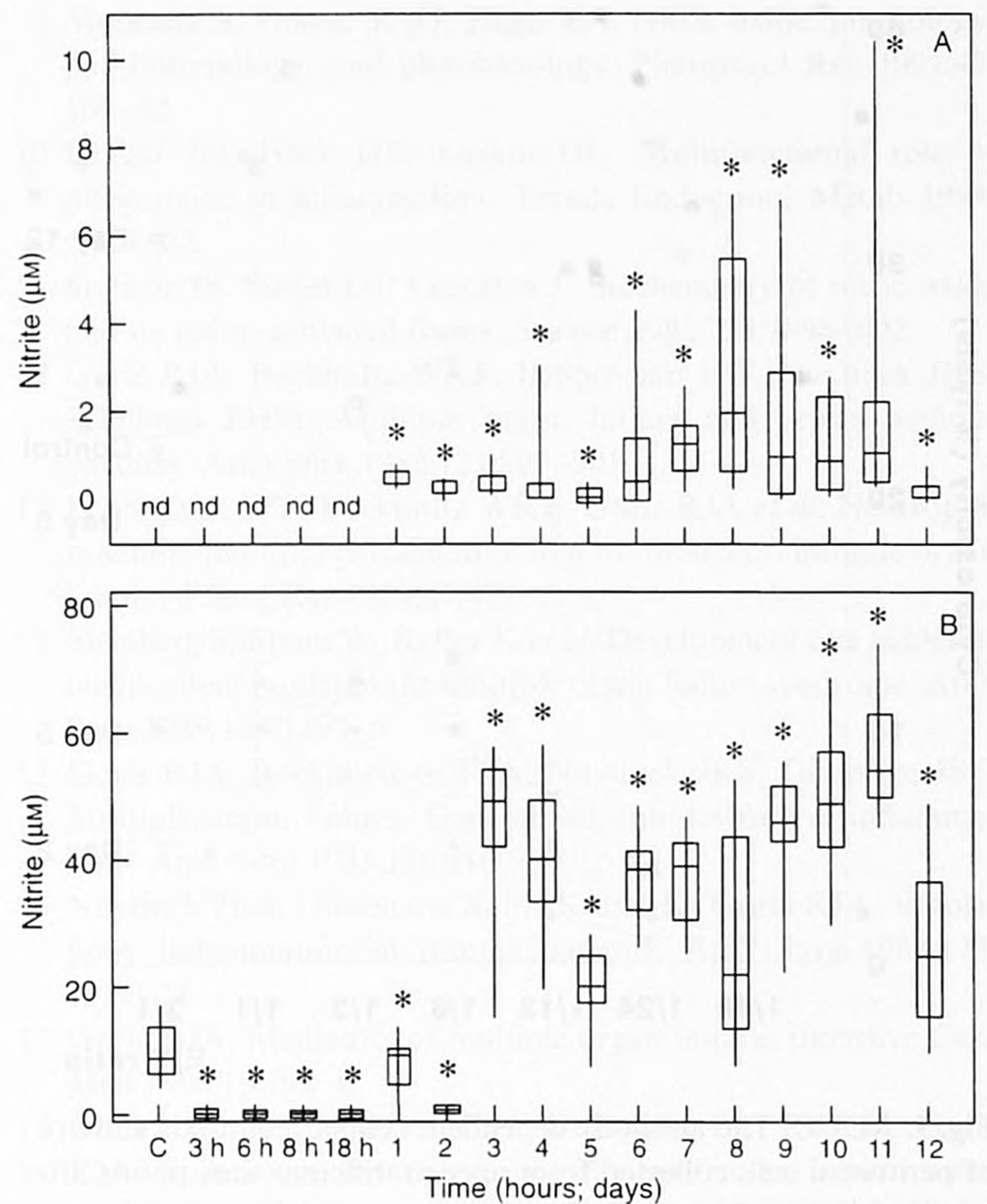


Fig. 4. Nitrite production by peritoneal cells. Non-stimulated (A) and LPS-stimulated (B) *in vitro* release of nitrite by mouse peritoneal cells, harvested at different time points after intraperitoneal administration of zymosan and by cells derived from control animals (C). Data are from observations on individual cultures from 8 to 14 mice and are expressed as medians in box plots ranging from the 25th to 75th percentile. Bars indicate the 5th and 95th percentile. *Significant difference ($P < 0.05$ by Kruskal-Wallis test) between control and experimental groups.

an acute sterile peritonitis from which they seem to recover in the second phase (days 3-7). From day 7 onwards the animals reach the third phase and develop signs of MODS.

During the first phase the bactericidal and cytotoxic functions of the peritoneal macrophages are suppressed. Stimulated NO production is also significantly suppressed in this phase. Nitrogen radicals produced by macrophages play an important role in cytotoxicity and are thought to be cytostatic or cytotoxic for a variety of pathogens [23-29]. O_2^- is involved in intracellular killing, and production during this phase is not altered during the phase, as is the capacity of macrophages to ingest non-opsonized *S. aureus*. Redmond *et al.* [30] showed in mice an impairment in phagocytosis of non-opsonized pathogens 1 day after laparotomy, but this function was restored 3 days after surgery. In our model we did not find such a depression in phagocytic function after inducing trauma, but our first measurements were performed 2 days after administration of zymosan, thus possibly missing transient effects occurring at an earlier time point.

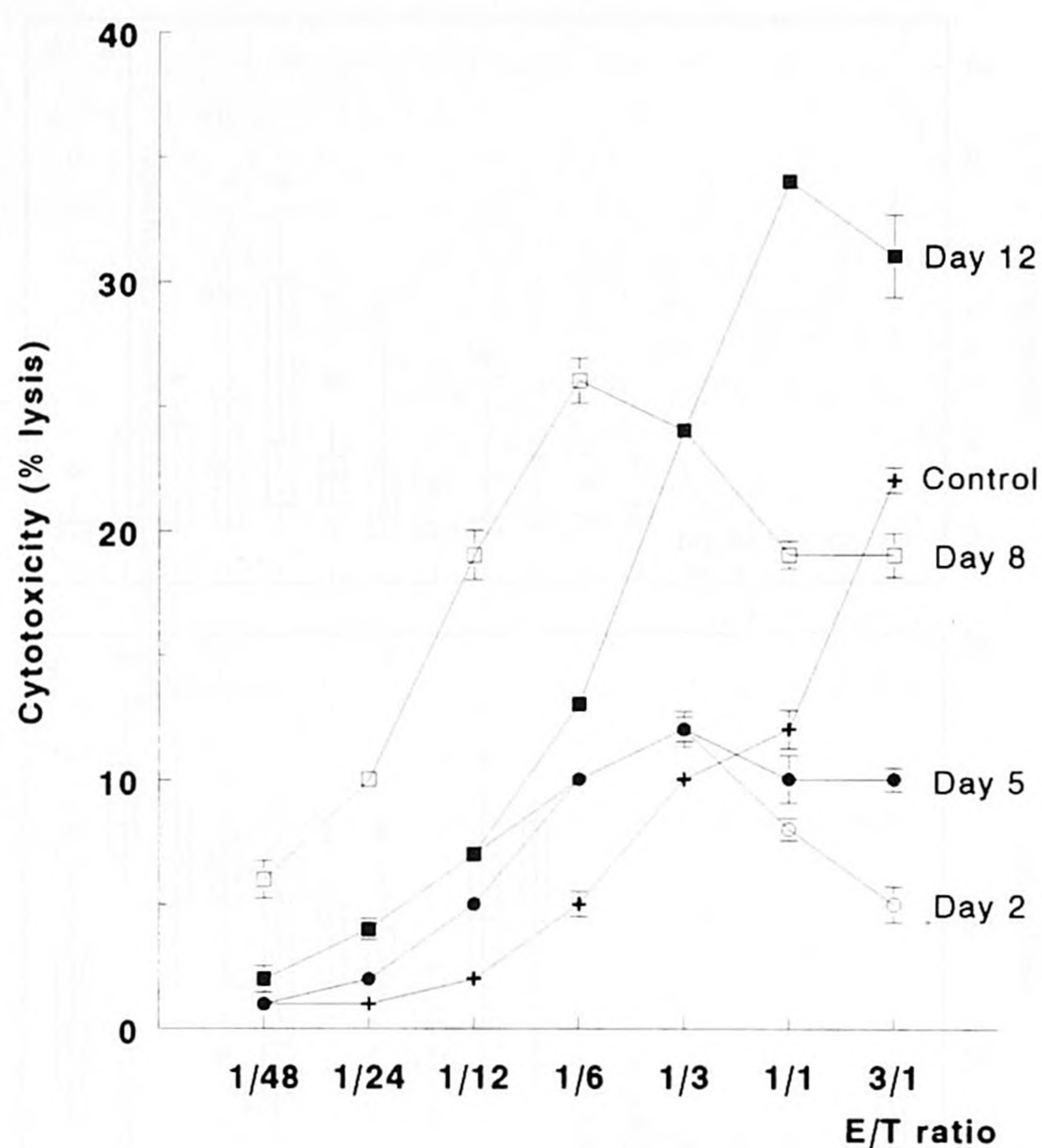


Fig. 5. ADCC. The antibody dependent cellular cytotoxic activity of peritoneal cells collected from mice at different time points after administration of zymosan. Results are expressed as mean values \pm SD of individual measurements performed in triplicate. This figure represents observations in a typical experiment which was repeated four times with essentially the same results.

During the second phase the macrophage functions seem to be restored or even enhanced. There appears to be a degree of overshoot with respect to the cytotoxic activity and the nitrogen radical production.

These processes continue in the third phase when the animals are developing MODS. When the first clinical signs of MODS develop (from day 7 onwards), production of oxygen and nitrogen radicals and cytotoxic activity are elevated in the animal model. Increased cytotoxic activity at a lower effector/target ratio could mean that there is a change in ligand-receptor interaction. Haisma *et al.* [31] found that macrophages, activated in different ways, which expressed equal numbers of Fc-receptors, differed in ADCC capacity. Furthermore, Fehr *et al.* [32] found that enhanced lytic activity induced by TNF is not necessarily due to enhanced Fc-mediated cell binding, and hypothesized that this may be due to activation of lytic mechanisms. Fan *et al.* [33] also found that incubation of peritoneal macrophages with TNF *in vitro* enhanced ADCC. Chicheportiche & Vassalli [34] found a reduction in Fc-RI and Fc-RIIb expression when macrophages were cultured in the presence of TNF- α . This finding supports the hypothesis of lytic mechanisms involved in the ADCC after incubation with TNF. In our model, FACS analysis showed no increase in Fc receptors on the effector cells (data not shown). We also found that in the third phase of our model stimulated TNF production by peritoneal cells *in vitro* is increased [35]. Thus, contact with target cells could possibly induce a rise in TNF and in this manner account for the elevated cytotoxic activity not through enhanced Fc-mediated cell binding, but possibly by lytic mechanisms. Another contribution to this mechanism could be the elevated production capacity of superoxide and nitric oxide by peritoneal macrophages. The reaction product of these two radicals, peroxynitrite (ONOO⁻), is also a highly reactive molecule. Peroxynitrite can decompose to hydroxyl radicals and these radicals may be involved in tissue damage [36, 37]. It is likely that when production of superoxide and

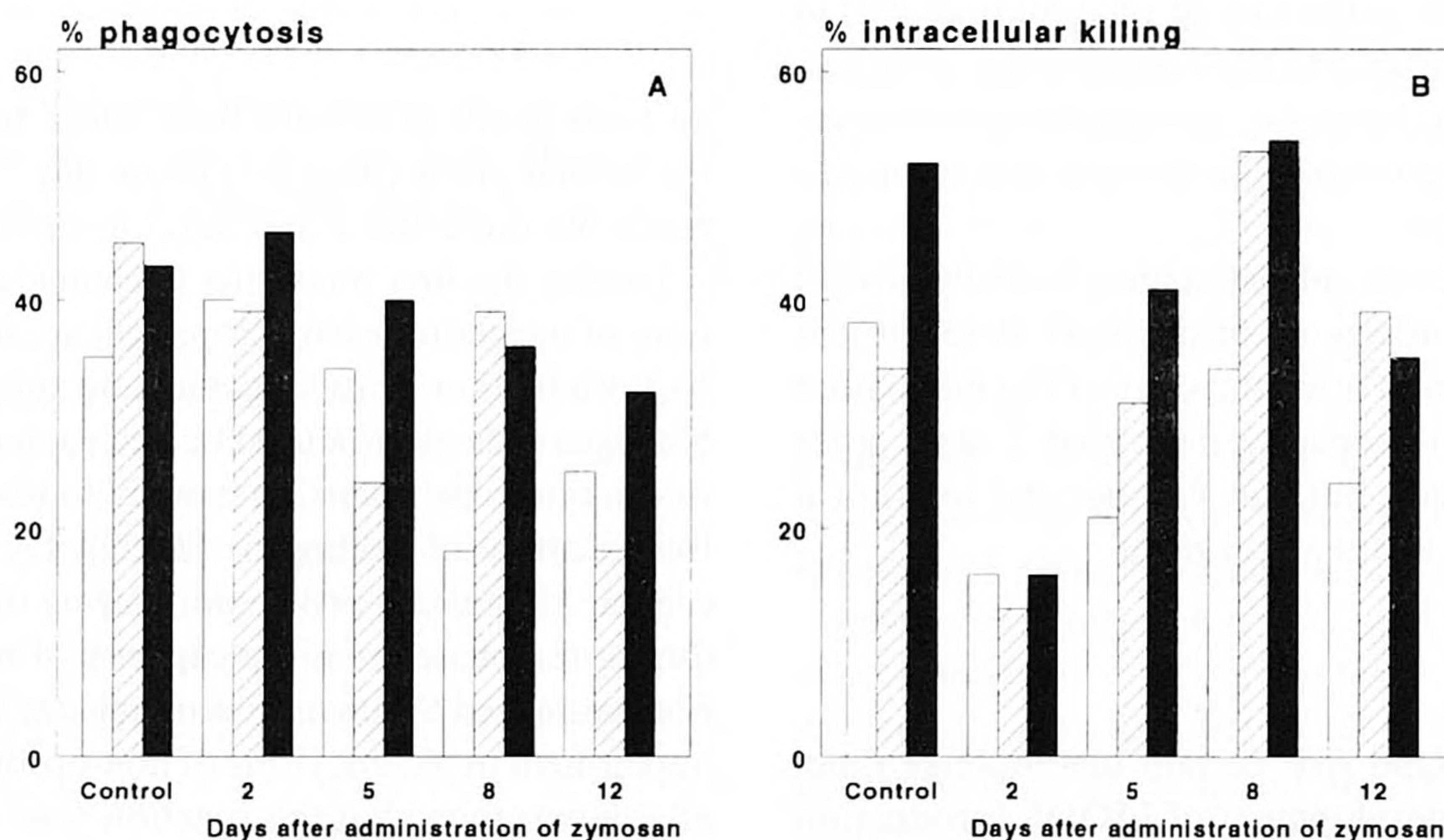


Fig. 6. *S. aureus* phagocytosis and intracellular killing. Phagocytic (A) and intracellular killing (B) capacity of cells from the control group and cells collected from animals at different time points after administration of zymosan. Open bars represent values obtained after 10 min of incubation, hatched bars represent values obtained after 20 min of incubation, and black bars represent values obtained after 30 min of incubation of cells. This figure represents observations in a typical experiment which was repeated four times with essentially the same results.

nitric oxide is high, the generation of peroxynitrite also rises, thus possibly inducing membrane damage and killing of targets. This could also affect the surrounding cells and tissues. On the other hand, Rubbo *et al.* [38] showed that nitric oxide has dual effects on lipid peroxidation; it can both stimulate superoxide-induced lipid oxidation and mediate oxidant-protective reactions in membranes when produced in high amounts. Whether this rise in radical production is detrimental or beneficial remains to be established.

Earlier experiments with germ-free animals [1] showed that the animals develop MODS despite the absence of bacteria, thus excluding the necessity of the micro-organism itself in the pathophysiology of MODS. This does not necessarily mean that bacteria are not involved in the mechanisms which contribute to the development of MODS in the present model.

A change in cell population phenotype could possibly account for the alterations, perhaps overactivation in the case of ADCC and radicals, of macrophage function. These changes in cytotoxic activity and radical production are concomitant with the onset and development of MODS. These findings seem to support our hypothesis that the macrophage is overactivated during the development of MODS, but whether these changes contribute to the development remains a topic for further investigation.

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