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Immune Cells Redox State from Mice with Endotoxin-induced Oxidative Stress. Involvement of NF-κB

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The immune cells, such as phagocytes and lymphocytes, which use reactive oxygen species (ROS) for carrying out many of their functions, need appropriate levels of intracellular antioxidants to avoid the harmful effect of oxidative stress. In previous studies, we have observed changes in several functions of those leukocytes from female BALB/c mice with lethal endotoxic shock caused by intraperitoneal injection of *Escherichia coli* 055:B5 lipopolysaccharide (LPS) (100 mg/kg), which were associated with high ROS production. In the present study, we have investigated the redox state of the above mentioned immune cells in that lethal endotoxic shock model measuring the oxidant/antioxidant balance through the following parameters: production of ROS, proinflammatory cytokine TNFα reduced glutathione (GSH), oxidized glutathione (GSSG), superoxide dismutase (SOD) and catalase (CAT) activities, malonaldehyde (MDA) and transcription factor NF-κB expression at different times after LPS injection. The results show an increase in ROS, TNFα and MDA production in both cell types, being higher in macrophages than in lymphocytes. GSSG/GSH ratio was increased in both macrophages and lymphocytes after LPS injection. With respect to the activity of the antioxidant enzymes SOD and CAT were decreased in both macrophages and lymphocytes. The activation of the transcription factor NF-κB was stimulated in macrophages and lymphocytes. These results point out that both lymphocytes and macrophages, which are able to play an important role in host response to endotoxin, show an oxidative stress thus contributing to the pathogenesis of this septic shock.

Keywords: Antioxidant defenses; Lymphocyte; Macrophage; Malonaldehyde; NF-κB; Oxidative stress

INTRODUCTION

Multiple organ system failure (MOSF) is the leading cause of mortality in medical and surgical intensive care units.[1] Sepsis-induced MOSF can be initiated by circulating lipopolysaccharide (LPS)[2] derived from the outer membrane of Gram-negative bacteria.[3] LPS activates the transcription and subsequent release of proinflammatory mediators, including cytokines and reactive oxygen species (ROS), through a receptor-mediated signaling pathway.[4] In inflammation and sepsis, excess amounts of ROS are produced during the respiratory burst process as a consequence of priming and stimulation of phagocytic leukocytes by cytokines, bacterial products or opsonized material.[5] The respiratory burst, characterized by the activation of the membrane-bound enzyme NADPH oxidase, forms superoxide anion, which dismutases to hydrogen peroxide.[6] Other radicals and oxidants are also produced, such as the hydroxyl radical, nitric oxide, peroxynitrite, singlet oxygen, hypochlorous acid, and other species capable of oxidizing a variety of compounds.[7]

Once produced, toxic oxidants are primarily directed to kill microorganisms. However, excess amounts of ROS can attack cellular components and lead to cell damage or death by oxidizing the membrane lipids, proteins, carbohydrates, and

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nucleic acids of host tissues. The host protects its tissues by strictly regulating the process of leukocyte activation[8] and an array of antioxidant enzymes and scavenger compounds help to limit oxidative damage to the site of infection.[9] How antioxidants cope with this increased oxidant load during sepsis has not been widely studied. A decrease in plasma antioxidant capacity in septic patients has been observed[10] as well as low antioxidant potential in septic patients, whereas it progressed toward normal levels in the survivors.[11] Thus, the responsible oxidative stress is the result of an imbalance between the generation of ROS and the antioxidant system in favor of the formers.

Since immune cell functions are specially linked to ROS generation[12] and are strongly influenced by the redox potential,[13] the oxidant/antioxidant balance is an important determinant of immune cell activity. The antioxidant levels in immune cells play a pivotal role protecting them against oxidative stress and therefore preserving their adequate function.[14] Thus, an optimal immune response requires proper levels of antioxidant enzymes and scavengers.[15] Antioxidant levels like those of reduced-glutathione (GSH), which plays an essential role in very important biological processes such as DNA synthesis, enzymatic reactions, neurotransmitter release or carcinogen detoxification[16] and protects against the ROS attack, show a positive effect on activation of T cells and other immune functions.[15] The antioxidant defense systems include enzymes like SOD and CAT that interact directly with ROS to neutralize them, and in immune cells are sensitive indicators of the antioxidant system deficiency and may be used, in addition to other markers, to follow oxidative stress progression.[17]

Transcription factors of the nuclear factor kappa B (NF-κB)/Rel family are ubiquitous and susceptible to regulation by changes in the intracellular reduction/oxidation state.[18] NF-κB binds to DNA, and has been identified in the promoter region of many mammalian genes. Typically, genes with NF-κB promoter sites encode cell adhesion molecules, immunoreceptors (T-cell receptor, MHC Class I and II, IL-2 receptors) and cytokines such as IL-2, IL-6, IL-8, β-interferon and mainly TNFα[19] TNFα is a key cytokine in endotoxic shock and contributes to pathophysiological changes associated with ROS production.[20] In view of this broad range of target genes, it is not surprising that NF-κ B is considered to be a crucial regulator of the immune system[21] with a very important role in pathologies associated to oxidative stress situations,[22] and it is considered a good therapeutic target.[23]

The excess of ROS causes peroxidation which can be measured through the lipoperoxide malonaldehyde test (MDA-TBARS).[24] Moreover, the increase of MDA levels could act as oxidative mediator.[25]

Although activated macrophages are widely recognized as cells that play an important role in inflammatory processes, recent work suggests an important role of lymphocytes in this process.[26–28] In order to find out which changes occur in the redox state of macrophages and lymphocytes in an oxidative stress situation such as the inflammatory processes associated with septic shock, we have measured in both cells from mice with lethal endotoxic shock the levels of ROS, the proinflammatory cytokine TNFα, several antioxidant defenses such as GSH, SOD and CAT, the levels of MDA and the changes in the expression of NF-κB.

MATERIALS AND METHODS

Materials

LPS (*Escherichia coli* 055:B5), EGTA, phorbol myristate acetate (PMA), HEPES, EDTA, phenylmethylsulfonyl fluoride, aprotinin, leupeptin, pepstatin, NaN_3, Nonidet P-40, glycerol, Tris, glycine, 1-4 dithioerythritol, L-glutamine, NADPH, glutathione reductase, 5-5′-dithiobis-(2-nitrobenzoic acid), thiobarbituric acid, pyrogallol, MDA, superoxide dismutase and trypsin were purchased from Sigma (St Louis, MO, USA). Culture plates of 96 wells were obtained from Costar (Cambridge, MA, USA) and of six wells from Corning Glass (Corning, NY, USA). Trypan blue, hydrogen peroxide and H_3PO_4 were obtained from Merck (Darmstadt, Germany). Acetonitrile were purchased from Carlo Erba (Milano, Italy). Tumor necrosis factor α (TNFα) immunoassay was obtained from Endogen (Woburn, MA, USA). RPMI 1640 medium and calf serum were purchased from Gibco (Paisley, Scotland, UK). 2′7′-dichlorofluoresceine (DDF-DA) was obtained from Molecular Probes (Eugene, Oregon, USA). K_2HPO_4, KCl, NaCl, Na_2HPO_4 and NaOH were purchased from Panreac (Barcelona, Spain).

Animals

Adult female BALB/c mice (Mus musculus) (Harlan Interfauna Ibérica, Barcelona, Spain), were maintained at a constant temperature (22 ± 2°C) in sterile conditions on a 12 h light/dark cycle and fed Sander Mus pellets (Panlab L.S. Barcelona, Spain) and water *ad libitum*. The animals used did not show any sign of malignancy or other pathological processes. Mice were treated according to the guidelines of the European Community Council Directives 86/6091 EEC. Although we have previously observed that the oestrous cycle phase of the mice has no effect on this experimental assay, all females used in the present study were at the beginning of dioestrous.

Experimental Protocol

A group of eight animals was used. The endotoxic shock was induced by intraperitoneal injection of *E. coli* LPS (055:B5) at a concentration of 100 mg/kg.[29] Each animal was injected with LPS between 9:00 and 10:00 a.m.

Mortality Experiment

In order to confirm that, as in our previous work, this injection of LPS produces an irreversible and lethal endotoxic shock, a group of 20 mice was used to observe mortality after endotoxin administration.

Collection of Cells

At 0, 2, 4, 12 and 24 h after LPS injections, peritoneal suspensions were obtained without sacrificing the mice by a procedure previously described.[30] Briefly, 3 ml of Hank's solution, adjusted to pH 7.4, were injected intraperitoneally, then the abdomen was massaged and the peritoneal exudate cells were collected allowing recovery of 90–95% of the injected volume containing lymphocytes and macrophages, which were identified by morphologic and cytometric assay. Peritoneal cells were incubated with Hank's solution in plates of 96 wells or of 6 wells or eppendorf tubes for 2 h to allow macrophages to form a monolayer, thus being separated from lymphocytes.[31]

Quantification of ROS

The ROS production was measured by flow cytometry using dichlorodihydrofluoresceine diacetate (DDF-DA) as a probe since it is oxidized in the cytoplasm by ROS to 2′7′-dichlorofluoresceine (DCF), which is a highly fluorescent compound. Aliquots of 200 µl of the peritoneal suspension (adjusted to 10^6 cells/ml) were centrifuged for 10 min at 1500 rpm and 4°C. The supernatants were discarded and the pellets were resuspended in 200 µl of Buffer A (Hank's medium without Ca^{+2} and Mg^{+2} and with EGTA 1 mM). The samples were incubated with 2 µl of DDF-DA (0.5 mM) for 15 min at 37°C. After incubation, 40 µl of phorbol miristate acetate (positive control) and 40 µl of Buffer A were added to the stimulated and the control samples, respectively. Samples were incubated for 15 min at 37°C and were then analyzed using a FACScan flow cytometer (Becton Dickinson, San Diego, USA). The results were expressed as fluorescence units (F.U.).

TNFα Measurement

The release of mouse tumor necrosis factor alpha (TNFα) was determined in macrophage and lymphocyte culture supernatants. Aliquots of 2×10^5 macrophages or lymphocytes/0.2 ml/well were incubated in plates of 96 wells with RPMI-1640 medium without phenol red and with L-glutamine and 10% heat-inactivated (56°C, 30 min) calf serum. After 24 h incubation, plates were centrifuged and the TNFα production was quantified in the supernatants using a mouse TNFα immunoassay with recombinant mouse TNFα a minimum detectable dose of mouse TNFα of 10 pg/ml and a limit of procedure of up to 1500 pg/ml.

Glutathione Determination

Total glutathione and GSSG were assayed by the method of Tietze[32] by monitoring the change in absorbance at 412 nm in the presence of 0.6 mM-5,5′-dithiobis-(2-nitrobenzoic acid), 0.21 mM-NADPH and 0.5 units of glutathione reductase (GR)/ml of assay mixture in 50 mM-phosphate buffer, pH 7.4. GSH and GSSG values were corrected for spontaneous reaction in the absence of sample. Leukocytes, adjusted to 1×10^6 cells (macrophages or lymphocytes)/ml of Hank's solution, and aliquots of 0.2 ml were used. The assay was performed at 25°C. Protein concentration was measured using the biuret method. The results are expressed as (µmol/g prot).

Superoxide Dismutase (SOD) and Catalase (CAT) Determination

Aliquots of 0.2 ml of macrophages or lymphocytes adjusted to 1×10^6 cells/ml of Hank's solution were homogenized and sonicated over ice. The sonicated homogenates were centrifuged 20 min at 3200*g* at 5°C, and the supernatants were used to measure SOD by quantifying the inhibition of pyrogallol autoxidation at 420 nm[33] (one unit of SOD is the amount of enzyme capable of inhibiting the rate of pyrogallol oxidation by 50%), and to measure CAT following the disappearance of H_2O_2 at 240 nm.[34] The results are expressed as µmoles H_2O_2/min mg prot for CAT activity, and as U/mg prot for SOD activity. Protein concentration was measured using the biuret method.

Peroxidation (MDA-TBARS)

At aliquots of 0.5 ml of macrophages or lymphocytes adjusted to 1×10^6 cells/ml of Hank's were added 0.5 ml H_3PO_4 and 25 µl of 2- thiobarbituric acid (TBA, 6 mg/ml). Thereafter, the samples were incubated in a bath at 95°C during 1 h. After this incubation, 150 µl of neutralizing solution (500 µl NaOH 1 N + 4.5 ml of acetonitrile) was added, and centrifuged at 4°C, 13000 rpm, for 5 min.

The MDA-TBARS determination in cells was carried out by high-performance liquid

chromatography (HPLC) and UV spectrophotometric detection at 532 nm using a Waters liquid chromatograph and a reverse-phase Novapak C18 column (Waters). As mobile phase, acetonitrile/water (70/30 by vol) containing K_2HPO_4 50 mM, pH 6.8 was used. Further filtration was performed with a 0.5 μm FHUP filter. The flow rate of the mobile phase was adjusted to 0.4 ml/min. MDA-TBARS working standards were prepared fresh daily in mobile phase. The results are expressed as nmoles/mg prot. Protein concentration was measured using the biuret method.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared by the mini-extraction procedure of Schreiber *et al.*[35] with slight modifications. The cells, macrophages or lymphocytes, were plated at a density of 10^7 cells/well in six-well plates, washed twice with ice-cold phosphate-buffered saline/0.1% bovine serum albumin, and scrapped off the dishes. The cell pellets were homogenized with 0.4 ml of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM NaN_3). After 15 min on ice, Nonidet P-40 was added to a final concentration of 0.5%, the tubes were gently vortexed for 15 s, and the nuclei were sedimented and separated from the cytosol by centrifugation at 12,000*g* for 40 s. Pelleted nuclei were washed once with 0.2 ml of ice-cold buffer A, and the soluble nuclear proteins were released by adding 0.1 ml of buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 25% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM NaN_3). After incubation for 30 min on ice, followed by centrifuging for 10 min at 14,000 rpm at 4°C, the supernatants containing the nuclear proteins were harvested. The protein concentration was determined by the biuret method, and aliquots were stored at −80°C for later use in EMSA. Aliquots of 50 ng of the double-stranded consensus oligonucleotide was end-labeled with [γ-^{32}P]-ATP by using T4 polynucleotide kinase. Standard binding reactions were performed by incubating 10 μg of nuclear extracts in 20 μl of 10 mmol/l Tris–HCl, pH 7.5, containing 100 mmol/l NaCl, 1 mmol/l dithiothreitol, 1 mmol/l EDTA, 10 mmol/l $MgCl_2$, 20% (v/v) glycerol, and 50000 dpm of ^{32}P-labeled NF-κB oligonucleotide (approximately 1 pmol), for 20 min at room temperature. To ensure specificity of probe binding, certain experiments were conducted in presence of a 100-fold molar excess of unlabeled (cold) NF-κB consensus oligonucleotide (data not shown). After incubation, the samples were loaded onto a 4% polyacrilamide gel and run at a constant current of 100 V in 0.5 × Tris–borate–EDTA (TBE) at 4°C. Gels were dried and placed on film at −80°C.

Expression of the Results and Statistical Analysis

The data are expressed as the mean ± standard deviation (SD) of the values from the number of experiments shown in the tables and figures. The data were analyzed by two-way repeated measures analysis of variance (ANOVA) in the different groups of mice at 0, 2, 4, 12 and 24 h after LPS injection. The Student Newman Keuls test with a minimum level of significance set at $p < 0.05$ were used for post-hoc comparisons.

RESULTS

Table I shows the accumulated percentage of mortality at different times after administration of LPS. The results show that the percentage mortality was 100% at 30 h.

Figure 1 shows the ROS production, at 0, 2, 4, 12 and 24 h after LPS injection, in macrophages and lymphocytes from peritoneum. The results show a significant increase at all times respect to 0 h, the peak being at 24 h. In all cases ROS production was higher in macrophages than lymphocytes.

The results of TNFα production at 0, 2, 4, 12 and 24 h after LPS injection by peritoneal macrophages and lymphocytes are shown in Fig. 2. In macrophages the levels were increased at all times after LPS injection, while in lymphocytes the levels of TNFα increased at 2 h after LPS injection. For both cell types, the highest levels of this proinflammatory cytokine was at 2 h, and the levels were higher in macrophages than in lymphocytes (significantly at 2, 4, 12 and 24 h).

The concentrations of GSH, GSSG as well as the GSSG/GSH ratio in macrophages and lymphocytes corresponding to animals at 0, 2, 4, 12 and 24 h after LPS injection are shown in Table II. The GSH content in lymphocytes was increased significantly at 2 and 4 h after LPS injection, while in macrophages it was decreased significantly at 4, 12 and 24 h. The GSSG/GSH ratio was increased in both types of cells at all times after LPS injection, and this increase was higher in macrophages than in lymphocytes.

TABLE I Mortality of animals after LPS injection

Time	Percentage of mortality (h)
0–12	0
13–20	0
20–24	12
24–30	100

Each value represents the accumulative percentage of mortality of animals after LPS injection.

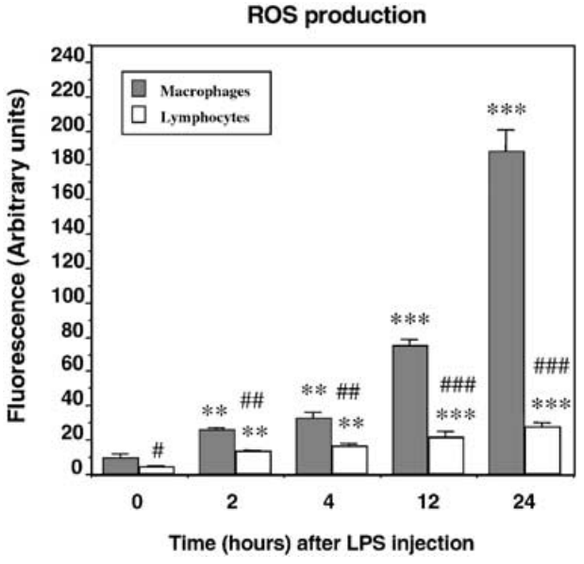

FIGURE 1 ROS production in peritoneal macrophages and lymphocytes. In all cases, the cells were obtained at 0, 2, 4, 12 and 24 h after injection. Each column represents the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays. $^{**}p < 0.01$ and $^{***}p < 0.001$ with respect to the corresponding values at 0 h. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ comparing the results of lymphocytes with those of macrophages.

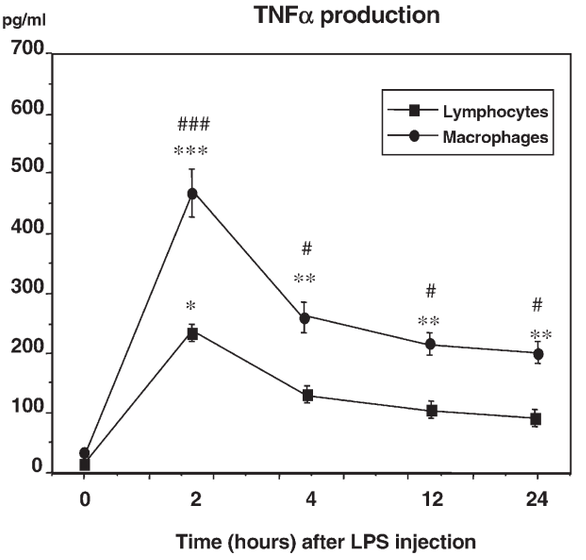

FIGURE 2 TNFα production in peritoneal macrophages and lymphocytes. In all cases, the cells were obtained at 0, 2, 4, 12 and 24 h after injection. Each column represents the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ with respect to the corresponding values at 0 h. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ comparing the results of lymphocytes with those of macrophages.

Figures 3 and 4 show the superoxide dismutase (SOD) and catalase (CAT) activity, respectively, in macrophages and lymphocytes at the different times (0, 2, 4, 12 and 24 h) after LPS injection. The SOD activity was decreased at 4, 12 and 24 h after LPS injection for macrophages and at 24 h for lymphocytes, the values being higher in lymphocytes than macrophages at 4 and 12 h. The CAT activity (Fig. 4) was decreased at all times. In control samples CAT activity was higher in macrophages than lymphocytes.

The endogenous levels of MDA (Fig. 5) were increased at 2, 4, 12 and 24 h after LPS injection in macrophages and at 4, 12 and 24 h in lymphocytes. The levels were higher in macrophages than in lymphocytes.

The activation of NF-κB *in vivo* in macrophages and lymphocytes at 1.5 h after LPS injection (Fig. 6) (time chosen after previous studies) is shown for both types of cells, with the density being higher in macrophages than in lymphocytes.

DISCUSSION

Immune cells, such as macrophages and lymphocytes are particularly sensitive to oxidative stress

TABLE II Levels of GSH, GSSG, and of the ratio GSSG/GSH (μmol/g prot) in macrophages and lymphocytes at different times after LPS injection

Time after LP injection (h)	GSH	GSSG	GSSG/GSH
Macrophages			
0	$25 \pm 3^{\#\#}$	1.8 ± 0.1	0.05 ± 0.005
2	$24 \pm 2^{\#\#}$	$1.9 \pm 0.2^{\#\#}$	$0.08 \pm 0.004^{***\#}$
4	$14 \pm 1^{***\#\#\#}$	$1.8 \pm 0.3^{\#\#\#}$	$0.12 \pm 0.004^{***\#}$
12	$13 \pm 1^{***\#\#\#}$	$1.8 \pm 0.3^{*\#\#}$	$0.14 \pm 0.005^{***\#}$
24	$12 \pm 1^{***\#\#\#}$	$2.0 \pm 0.2^{*\#\#}$	$0.18 \pm 0.004^{***\#\#\#}$
Lymphocytes			
0	45 ± 3	1.4 ± 0.2	0.03 ± 0.003
2	$58 \pm 7^{**}$	$3.4 \pm 0.4^{***}$	$0.06 \pm 0.005^{***}$
4	$72 \pm 8^{***}$	$5.4 \pm 0.4^{***}$	$0.07 \pm 0.004^{***}$
12	47 ± 5	$3.9 \pm 0.4^{***}$	$0.07 \pm 0.004^{***}$
24	40 ± 4	$3.4 \pm 0.3^{***}$	$0.09 \pm 0.006^{***}$

The results are the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays. LPS (cells from animals injected with 100 mg/kg of LPS). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ with respect to the corresponding values at 0 h. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$, comparing, the results of lymphocytes with those of macrophages.

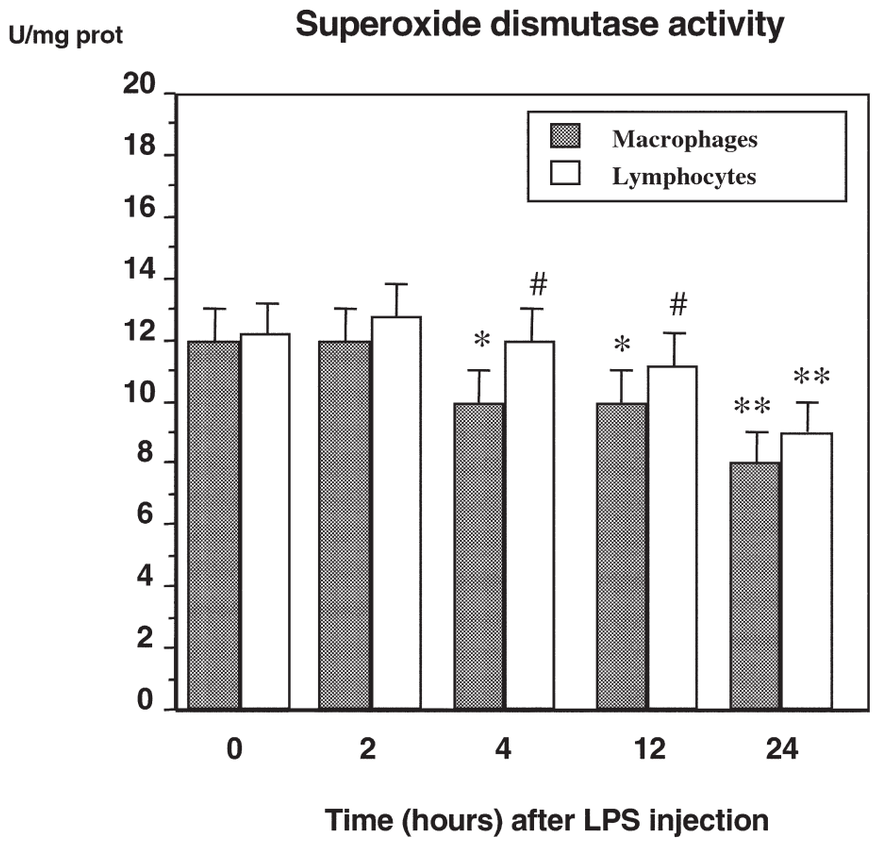

FIGURE 3 Superoxide dismutase activity in peritoneal macrophages and lymphocytes. In all cases, the cells were obtained at 0, 2, 4, 12 and 24 h after injection. Each column represents the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays. $^{*}p < 0.05$ and $^{**}p < 0.01$ with respect to the corresponding values at 0 h. $^{\#}p < 0.05$ comparing the results of lymphocytes with those of macrophages.

because of the high percent of polyunsaturated fatty acids present in their plasma membranes and a higher production of ROS, which is part of their normal function.[15] Thus, the pro-oxidant/antioxidant balance is critical for immune cell function because it maintains cell membrane integrity and functionality and controls signal transduction and gene expression.

Immune cells in general, and particularly macrophages and lymphocytes, play an essential role in the immune response associated with the host inflammatory and infectious processes. In this study, we have demonstrated the oxidative stress of macrophages and lymphocytes from mice with lethal endotoxic shock caused by intraperitoneal injection of *E. coli* (100 mg/kg). These cells produce and release ROS and the proinflammatory cytokine TNFα in high amount after LPS injection, and this response is more marked in macrophages than in lymphocytes. This effect could be due that lymphocytes contain higher amounts of ascorbic acid than macrophages.[27] Both, ROS and TNFα act as a signaling elements in the pathophisiology of the systemic inflammatory response in critically ill patients.[4] Although macrophages have been considered as the principal cell in response to endotoxin, releasing proinflammatory mediators such as ROS and TNFα, in the present work we have demonstrated that lymphocytes also play an important role in the inflammatory processes, contributing to the oxidative stress implicated in the pathogenesis of endotoxic shock.

To assess the redox state of macrophages and lymphocytes during endotoxic shock, the intracellular levels of GSH, GSSG, SOD and CAT have been measured. These antioxidants seem to play a crucial role preserving the function of immune cells by protecting them against oxidative stress.[15] As regards the levels of GSH, which is included in the first line of defense against oxidative processes,[36] they decrease at all times after LPS injection in macrophages, while in lymphocytes they increase at 2 and 4, showing a marked ability of these cells to concentrate antioxidants. However, the levels of GSSG increase in macrophages at 12 and 24 h after LPS injection and in lymphocytes at all times after LPS administration. Thus, the GSSG/GSH ratio increased in both types of cells, showing a higher increase in macrophages than in lymphocytes. These

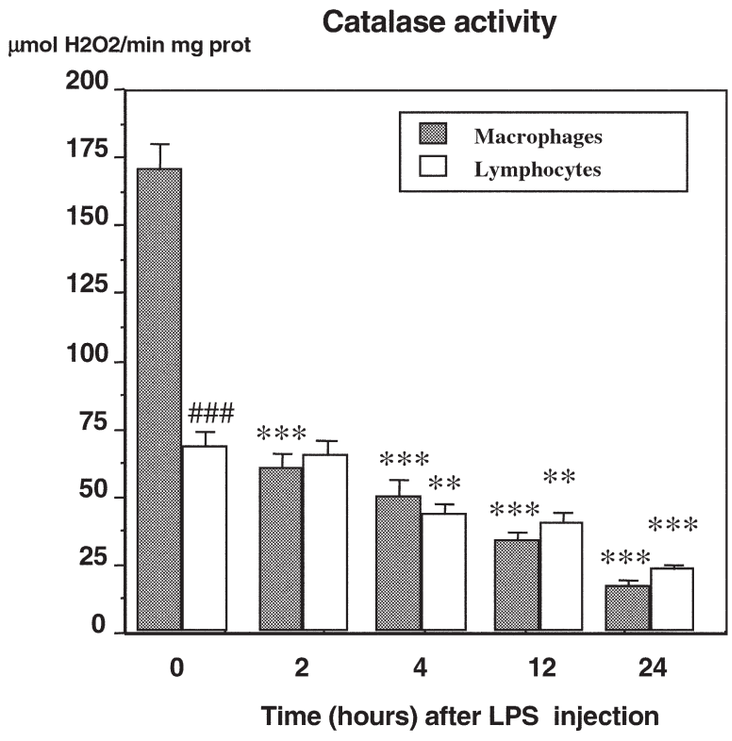

FIGURE 4 Catalase activity in peritoneal macrophages and lymphocytes. In all cases, the cells were obtained at 0, 2, 4, 12 and 24 h after injection. Each column represents the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays. $^{**}p < 0.01$ and $^{***}p < 0.001$ with respect to the corresponding values at 0 h. $^{\#\#\#}p < 0.01$ comparing the results of lymphocytes with those of macrophages.

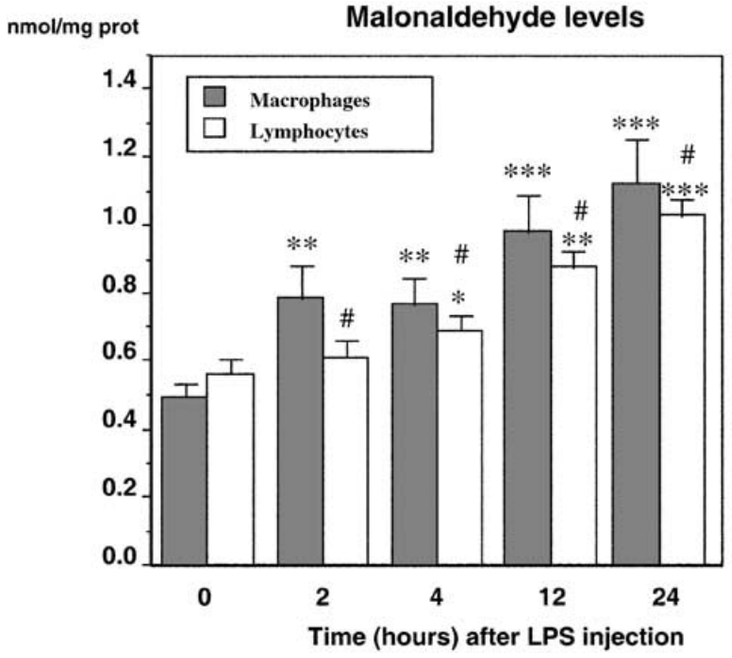

FIGURE 5 Malonaldehyde levels in peritoneal macrophages and lymphocytes. In all cases, the cells were obtained at 0, 2, 4, 12 and 24 h after injection. Each column represents the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ with respect to the corresponding values at 0 h. $^{\#}p < 0.05$ comparing the results of lymphocytes with those of macrophages.

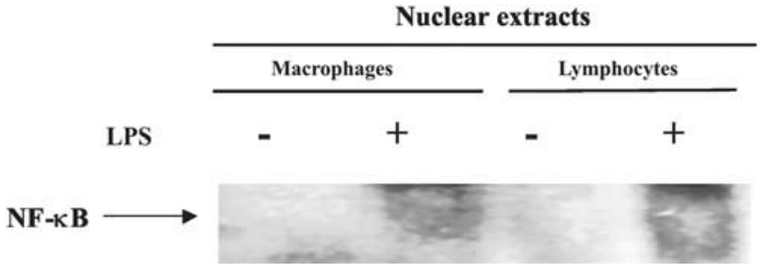

FIGURE 6 NF-κB activation in peritoneal macrophages and lymphocytes. In all cases the cells were obtained at 1.5 h after LPS injection. One representative experiment of six is shown. To ensure specificity of probe binding, certain experiments were conducted in presence of a 100-fold molar excess of unlabeled (cold) NF-κB consensus oligonucleotide (data not shown).

results could be related with the values obtained in ROS and TNFα production, parameters that were higher in macrophages than lymphocytes. Thus, macrophages seem to use intracellular antioxidants to avoid the damage caused by ROS while lymphocytes mainly accumulate antioxidants like a protective mechanism against peroxidation death.

In relation to the activity of the antioxidant enzyme SOD, there is a decrease of their activity at 4, 12 and 24 h in macrophages, and at 24 h in lymphocytes. These results are in agreement with other studies which demonstrate that SOD decreases in situations of oxidative stress.[17] With respect to the activity of CAT, it decreased at all times after LPS injection.

Lipid peroxidation results from an imbalance between oxidants and antioxidants that occurs in oxidative stress conditions.[37] For this reason we have evaluated the levels of MDA in macrophages and lymphocytes, observing that there was an increase at all times after LPS injection. Moreover, we have demonstrated that both macrophages and lymphocytes are target of the oxidative stress caused by endotoxin. Another report has previously shown an increase in plasma levels of lipoperoxides in a rat endotoxin shock model.[24] Moreover, the increased MDA levels could act as oxidative mediators.[25]

Although some transcription factors are cell-specific, other, such as NF-κB, are ubiquitous. Importantly, NF-κB governs the expression of genes that encode cytokines such as TNFα, chemokines, cell adhesion molecules, growth factors, and some acute phase proteins in several diseases.[38] In the present work, we have observed *ex vivo* an activation of this transcription factor in both types of cells after LPS administration, demonstrating again that both cells are involved in the oxidative stress caused by endotoxin. In fact, a depletion of reduced GSH and subsequent increases in cytosolic GSSG in response to oxidative stress causes rapid ubiquitination and phosphorylation and thus subsequent degradation on the inhibitory κB (IκB) complex, which is critical step for NF-κB activation.[39,40] Moreover, this fact is correlated with the high production of TNFα in those immune cells.

Although LPS exhibits a variety of biological functions, it is evident that it induces an oxidative state in immune cells, not only in macrophages, but also in lymphocytes as well. Thus, both immune cell types produce in response to LPS high quantities of proinflammatory mediators, use antioxidants in high amounts, and contribute to induce oxidative stress increasing peroxidation and activation of the NF-κB. Since the animal with this endotoxic shock shows a mortality of 100% at 30 h, the parameters studied in the present work seem to be important markers of the oxidative stress and animal survival. Moreover, the intensity and duration of an inflammatory process depends on the local balance between oxidants and antioxidants, mainly in the immune system, in response to endotoxins. Thus, these results open the door to knowledge of the possible targets of the therapy against situations involving oxidative stress, as well as to the use in them of antioxidants as therapeutic tools.

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