MICROBIAL PATHOGENICITY

In-vivo induction of apoptosis in murine lymphocytes by bacterial lipopolysaccharides

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Summary. The effect of bacterial lipopolysaccharide (LPS) on the lymphoid organs in C3H/HeN and C3H/HeJ mice was investigated. In C3H/HeN mice, LPS induced apoptosis, characterised by morphological nuclear condensation and DNA fragmentation resulting in thymic atrophy. Similar but less severe changes were also observed in the spleen and lymph nodes. In C3H/HeJ mice, only a slight depletion of lymphocyte numbers was observed in the lymphoid organs. The plasma endotoxin levels were dependent on the LPS dose regardless of mouse strain. On the other hand, the plasma TNF- α levels were significantly elevated in C3H/HeN mice 1 h post-injection and the time course of plasma corticosterone concentration correlated well with the development of apoptosis. These findings suggest that TNF- α and corticosterone may play an important role in LPS-induced apoptosis of lymphocytes.

Introduction

Lipopolysaccharide (LPS) from the cell walls of gram-negative bacteria is capable of eliciting a wide variety of pathophysiological effects such as shock, tissue injury and death in man and animals.^{1,2} Furthermore, LPS is a potent stimulant of the host immune system.³ LPS is a mitogen that stimulates proliferation and differentiation of B-lymphocytes and initiates macrophage activation, thus enhancing immune responses.^{4–6} However, little is known about the effects of LPS on lymphoid tissue *in vivo* except for several reports of depletion of thymocytes in mice given injections of LPS.^{7–9} More recently, Zhang *et al.*¹⁰ have indicated that this depletion of thymocytes could be attributed to apoptosis. They also suggested that apoptosis was mediated by adrenocortical hormones and TNF- α .

In this study, the in-vivo effect of LPS on the lymphoid organs of LPS-sensitive C3H/HeN and LPS-resistant C3H/HeJ mice—which differ in the *lps* gene^{11–14}—was investigated.

Materials and methods

LPS

LPS from *Escherichia coli* O55:B5 extracted by the Westphal method was purchased from Difco. LPS was

suspended in LPS-free saline (Otsuka, Tokyo, Japan) at various concentrations.

Animals and inoculation methods

Seventy-five 13-week-old female mice of LPS-sensitive strain C3H/HeN and 40 13-week-old female mice of LPS-resistant strain C3H/HeJ were purchased from Japan SLC, Inc., Shizuoka, Japan. C3H/HeN mice were divided into two groups. According to the results of preliminary determinations in C3H/HeN mice showing that the LPS LD50 was 3 mg/kg body weight, mice in each group were given 1 (NL group) or 9 (NH group) mg/kg body weight by intravenous injection. C3H/HeJ mice were given 30 mg/kg body weight (LD50 was > 200 mg/kg body weight; group J). In each case, five animals were killed by exsanguination under ether anaesthesia at 1, 6, 12, 24 and 48 h and 5 days post-injection. Plasma samples were collected in LPS-free heparin by heart puncture and stored at -80°C until used. For the peripheral blood cell count, five mice/group were bled from the orbital sinus at various times under ether anaesthesia. Leucocytes, platelets and red blood cells were diluted with Türk solution, ammonium oxalate 1% solution and Hayem's solution, respectively, and counted in a haemocytometer. Mice of both strains given LPS-free saline served as controls.

Histopathological examination

For light microscopy, cerebrum, cerebellum, thymus, heart, lungs, intestines, spleen, mesenteric lymph

nodes, pancreas, kidneys and adrenal glands were removed and fixed in neutral buffered formalin 10%, 2- μ m-thick paraffin sections were made and stained with haematoxylin and eosin. For lipid detection, frozen sections of the livers of group NL were stained with Sudan III. For electronmicroscopy, small pieces of tissue fixed with formalin were post-fixed in osmium tetroxide 1.0% in 0.1 M phosphate buffer (pH 7.4) and embedded in epoxy resin (Quetol 812; Nissin EM Co. Ltd, Tokyo, Japan). Ultra-thin sections were double-stained with uranyl acetate and lead citrate and observed with a JEM-100S electronmicroscope (JEOL Ltd, Tokyo, Japan).

DNA fragmentation assay

Freshly isolated thymus, spleen and mesenteric lymph nodes at various stages post-injection were separated by wire mesh, collected in 0.1 M phosphate-buffered saline and washed three times. DNA from these cells was then extracted by a DNA extractor WB kit (Wako Pure Chemical Industries Ltd, Osaka, Japan). The extracted DNA was mixed with TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and subjected to electrophoresis in agarose 2% gel. Gels were stained with ethidium bromide 1 μ g/ml and visualised with UV light.

Endotoxin assay

Endotoxin levels in plasma were assayed by the kinetic turbidimetric method with the Limulus ES J test Wako (Wako Pure Chemical Industries Ltd), an endotoxin-specific *Limulus* amoebocyte lysate (LAL-ES) test. Briefly, endotoxin-free glassware was prepared by baking at 250°C for 2 h. Plasma samples were diluted 10-fold with Sample Pretreatment Solution (Wako Pure Chemical Industries Ltd) which contained Triton X-100 0.04% and heated at 80°C for 5 min. A 0.1-ml sample of test fluid was added to 0.1 ml of LAL-ES in a 10 $\times$ 75 mm glass tube. The test tube was set on the Toxinometer ET-201 after vortex mixing for a few seconds. The gelation time was measured by the Toxinometer ET-201 (Wako Pure Chemical Industries Ltd) and the concentrations were calculated with the LS-Toximaster (Wako Pure Chemical Industries Ltd), a data acquisition program for the Toxinometer. All operations were performed at 0°C.

Tumour necrosis factor (TNF)- α bioassay

TNF- α levels in plasma were assayed by the MTT tetrazolium cytotoxicity assay with the murine fibrosarcoma cell line WEHI 164 clone 28.¹⁵ Recombinant mouse TNF- α (Genzyme, Cambridge, USA) was used to prepare a standard curve. Results were presented as the mean of three determinations. The specificity was confirmed by inhibition by rabbit anti-mouse TNF- α antibody (Genzyme). The lower limit of detection was 1 pg/ml.

Corticosterone assay

Corticosterone levels in plasma were measured by HPLC with fluorimetric detection as described by Mason *et al.*¹⁶ The column (100 mm $\times$ 4.6 mm internal diameter) contained Nucleosil 5C₁₈ and the flow rate was 2.0 ml/min. Fluorescence was monitored with excitation and emission wavelengths set at 375 nm and 485 nm, respectively.

Statistical analysis

The statistical analysis was performed with Veterinarian's Software series (Bunido Publishing Co. Ltd, Tokyo, Japan).

Results

Clinical findings

At 6 h post-injection, mice of both groups showed clinical signs such as ruffled fur and reluctance to movement. Most animals of group NH died between 24 and 48 h. All the animals of group NL returned to normal by 48 h. Animals of group J showed no clinical signs. The number of peripheral blood leucocytes in all groups dropped to less than one-third of the pre-injection value within 1 h and returned to normal by 5 days.

Histopathological and electronmicroscopy findings

Groups NH and NL. In the thymus of mice of both test groups, as compared with controls, small foci of cortical lymphocytes showing karyorrhexis or pyknosis were observed at 6 h. The lesions gradually progressed and multifocal loss of cortical lymphocytes was observed at 24 h (fig. 1a). By electronmicroscopy, as compared with controls (fig. 2a), cortical lymphocytes showed marked condensation of nuclear chromatin (fig. 2b) or fragmentation of nuclei, or both. After 48 h, animals of group NL showed apparent thymic atrophy due to loss of cortical lymphocytes. In the spleen and mesenteric lymph nodes, similar but less severe changes were observed in lymphoid follicles (figs. 1b, c). In other organs, marked neutrophil infiltration occurred in the interalveolar septa of the lungs within 6 h, in the adrenal glands, cortical parenchymal cells showed loss of lipid droplets on and after 6 h and in the liver, hepatocellular necrosis or microthrombi, or both, developed after 6 h. Those changes lasted until the NH mice died; they disappeared on and after 12 h in the NL group. At 48 h, mice of group NL showed Sudan III-positive lipid deposition in perilobular hepatocytes.

Group J. Animals of group J showed a slight depletion of lymphocytes in the lymphoid organs at 6 h. The lesion did not progress and recovered to normal. In the lungs, the same lesion as that in groups

Fig. 1. Light microscopic appearance of: **a**, thymus; **b**, spleen; **c**, mesenteric lymph node of a NL mouse at 24 h. Haematoxylin and eosin stain. Bars, 60 μ m. Arrows indicate small foci of follicular lymphocytes showing pyknosis or karyorrhexis.

Fig. 2. Electronmicroscopic appearance of cortex of thymus of: **a**, control; **b**, NL mouse at 24 h. Bars, 2 μ m.

NH and NL was observed. Other organs showed no lesions.

DNA fragmentation

As shown in fig. 3a, DNA extracted from thymocytes of group NH mice showed a ladder of multiples of 190 bp, indicating the fragmentation of nucleosomal DNA by endonuclease. These fragmentations were detected to a small extent at 6 h and clearly

at 12 h. At 24 h, fragmentation was less clear. DNA fragmentation was also observed in the spleen and in the mesenteric lymph nodes (figs. 3b, c).

Endotoxin levels in plasma

As shown in fig. 4, the plasma endotoxin levels were high at 1 h, according to the dose of LPS and regardless of the mouse strains. They gradually decreased and, in groups NL and J, mostly disappeared by 5 days.

Fig. 3. DNA fragmentation of control and NL mice. a, Thymus: control cells show no fragmentation (lane 2), whereas cells of NL mouse begin to show a ladder of multiples of 190 bp at 6 h (3) and clearly at 12 h (4); at 24 h, DNA fragmentation appeared less clearly than at 12 h (5). Molecular size standards are shown in lane 1 (from the upper band: 955, 585, 341, 258, 141, 105 and 78 bp). Slight ladder formation was observed in: b, spleen and c, mesenteric lymph node.

Fig. 4. Endotoxin levels (mean and SD) in plasma of NH (●), NL (○) and J (□) mice.

TNF- α levels in plasma

As shown in fig. 5, groups NL and NH showed clear elevation of TNF- α activity only at 1 h. Group J did not show any significant TNF- α activity.

Corticosterone levels in plasma

As shown in fig. 6, plasma corticosterone levels in groups NH and NL were elevated at 1 h. In group NH, the level remained high until death, whereas, in group NL, it began to drop after 24 h and returned to normal at 48 h. The time course of changes in corticosterone levels in mice of group NL closely paralleled the development of histological changes in the lymphoid organs. Corticosterone levels decreased in accordance with the appearance of lipid deposits in the liver. In group J, the plasma corticosterone levels remained relatively low and returned to normal earlier. Control mice of both C3H/HeN and C3H/HeJ, which were

given LPS-free saline and underwent the same operation as LPS-treated groups, showed no elevation of corticosterone levels.

Discussion

This study showed that bacterial LPS induced depletion of lymphocytes in the lymphoid organs in LPS-sensitive mice but not in LPS-resistant mice. Electronmicroscopic and electrophoretic examination revealed that this change was due to apoptosis. Recently, Zhang *et al.*¹⁰ reported in-vivo induction of DNA fragmentation by LPS in the thymus but not in other lymphoid organs of young BALB/c mice. In the present study, apoptotic change was also detected in the spleen and lymph nodes in 13-week-old LPS-sensitive mice although the change was less severe than that in the thymus. Although the dose and the origin of

Fig. 5. TNF- α levels (mean and SD) in plasma of NH (●), NL (○) and J (□) mice.

Fig. 6. Corticosterone levels (mean and SD) in plasma of NH (●), NL (○), J (□) and control (C3H/HeN (Δ) and C3H/HeJ (▽)) mice.

LPS and the mouse strain differed in each study, our data suggest that the apoptotic change induced by LPS may not be restricted to immature thymocytes. In fact, similar apoptotic changes in lymphocytes were also detected in 7-week-old C3H/HeN mice after LPS injection (data not shown), indicating that the change occurred regardless of age.

The time course of plasma endotoxin concentrations showed no strain difference, whereas plasma TNF- α levels showed a significant difference between LPS-sensitive and LPS-resistant mice shortly after injection. Strain differences were also detected in both the peak

levels and the time taken for normalisation of elevated plasma corticosterone levels to occur. Within the network of cytokine, nerve and endocrine systems, early inflammatory cytokines such as TNF- α and interleukin (IL)-1, mainly produced by macrophages, stimulate the pituitary gland to release glucocorticoids.^{17,18} These glucocorticoids then inhibit the production of TNF- α ¹⁹⁻²² and IL-1²³⁻²⁶ and lead to recovery from inflammation. Recent reports have suggested that TNF- α or adrenal hormone, or both, play an important role in LPS-induced thymocyte apoptosis.^{10,27} In the present study, the changes of

plasma levels of TNF- α and corticosterone *in vivo* in LPS-sensitive mice were consistent with the above-mentioned cytokine-nerve-endocrine network theory and support the view that TNF- α or adrenal hormone, or both, are responsible for thymocyte apoptosis. Furthermore, the fact that LPS-resistant mice showed neither apoptosis nor changes of plasma TNF- α and corticosterone suggests that TNF- α and corticosterone are necessary for LPS-induced lymphocyte apoptosis.

Further studies should be conducted to elucidate the precise mechanism of the *in-vivo* apoptosis of lymphocytes induced by LPS. Brouckaert *et al.*²⁸ reported that TNF- α influenced the sensitivity of lymphocytes to glucocorticoid hormones. However, other cytokines and their co-operation with LPS should not be excluded. Recently Albina *et al.*²⁹ reported nitric oxide-mediated apoptosis in murine peritoneal macrophages *in vitro* after treatment with IFN- γ and LPS. Furthermore, the mechanism of apoptosis induced by LPS in the thymus may differ

from that in the other lymphoid organs, and lymphocyte populations that showed apoptosis in the spleen and lymph nodes should be identified.

Apoptosis plays an important role in the haemopoietic and immune systems during differentiation of erythroid progenitor cells, maturation of B-lymphocytes and target cell lysis by natural killer cells and cytotoxic T-lymphocytes.³⁰ It is controversial whether apoptosis of lymphocytes, especially in the thymus, is responsible for lethality during endotoxin shock. However, considering the essential role of T-lymphocytes in the immune system,³¹ it is reasonable to consider that a depletion of lymphocytes, especially in the lymphoid organs, may change immunological functions and may have a prognostic influence in endotoxin shock.

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