In Vivo Induction of Apoptosis (Programmed Cell Death) in Mouse Thymus by Administration of Lipopolysaccharide

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In vivo administration of bacterial lipopolysaccharide to mice induced DNA fragmentation in the thymus. Fragmented DNA was confirmed by agarose gel electrophoresis and laser flow cytometry. DNA fragmentation was predominantly detected in the thymus of young mice, while it was undetectable in the spleen, bone marrow, and lymph nodes. DNA fragmentation in the thymus was roughly dependent on the dose of lipopolysaccharide injected and reached the peak about 18 h after the injection. The addition of lipopolysaccharide in vitro cultures of thymocytes did not cause DNA fragmentation, suggesting that lipopolysaccharide was unable to induce apoptosis of thymocytes directly. The injection of lipopolysaccharide induced no significant DNA fragmentation in adrenalectomized mice. The injection of anti-tumor necrosis factor alpha antibody together with lipopolysaccharide partially inhibited the appearance of DNA fragmentation in the thymus. On the basis of the fact that DNA fragmentation is one of the characteristics typical in apoptotic cell death, it was suggested that lipopolysaccharide could induce apoptosis in the mouse thymus in vivo. This apoptosis in the thymus might be mediated mainly by the adrenal hormones, but it is likely that tumor necrosis factor alpha might also participate in it.

Bacterial lipopolysaccharide (LPS) exhibits a variety of biologic activities, such as toxicity, adjuvanticity, anticomplementary activity, B-cell mitogenicity, and interferon-inducing activity (12, 13). It has been demonstrated that in vivo administration of LPS induces a loss of thymic weight and a decrease of thymic lymphocytes (1, 2, 15, 22, 23). The changes in thymus morphology consist mainly of the destruction of thymic lymphocytes in the cortex, whereas the medulla seems not to be affected. Rowlands et al. (15) and Adorini et al. (1) have reported that the phenomena in the thymus of mice injected with LPS are due to the necrosis of cortical thymocytes.

Cell death is now recognized to be of fundamental importance in many fields of biology. Particularly, the immune system provides good models for cell death. Cell death can generally proceed via necrosis or apoptosis (programmed cell death) (4, 9, 10). Necrosis is characterized by the formation of tubular lesions (pores) in the plasma membrane. On the other hand, apoptosis causes cell death in a way that differs morphologically and biochemically from necrosis.

The common core mechanism of apoptosis is DNA fragmentation and morphological lesions, such as condensation and fragmentation of nucleus and cytoplasm. Although thymic injuries induced by LPS were previously reported as the necrosis of thymic lymphocytes, the possibility that LPS might induce their cell death through apoptosis was raised. In the present study, we studied whether and how the administration of LPS into mice induces apoptosis in thymic lymphocytes in vivo.

MATERIALS AND METHODS

Animals. Female and male BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan) and used at 4 to 5 weeks of age. Fifteen-week-old BALB/c mice were also used.

Reagents. Propidium iodide and hydrocortisone were obtained from Sigma, St. Louis, Mo. Rat monoclonal antinice tumor necrosis factor alpha (TNF-α) antibody was purchased from UB, Inc., Lake Placid, N.Y.

LPS and administration. The LPS preparation extracted by the phenol-water method from Escherichia coli O111:B4 or Salmonella enteritidis was obtained from Difco Laboratories, Detroit, Mich. LPS from Klebsiella pneumoniae O3 was also prepared by the phenol-water method (19, 21). LPS from E. coli O111 was injected intraperitoneally (i.p.) unless otherwise stated. Mice were injected i.p. with various concentrations of LPS. Two or three mice per each experimental group were sacrificed at various days after injection, and the thymus, bone marrow, spleen, and inguinal lymph nodes were collected. The lymphoid organs were dissected to prepare single cell suspensions in 0.01 M phosphate-buffered saline (PBS) at pH 7.2.

Cell preparation and culture. The thymus was dissected in RPMI 1640 medium, and the cell suspension was passed through a nylon sieve. The cell suspension was washed and adjusted to a concentration of 2 × 10⁶/ml in RPMI 1640 medium supplemented with 10% fetal calf serum. One milliliter of the cell suspension was incubated in tissue culture plates with or without the addition of LPS (50 μg/ml) under a humidified 5% CO₂ atmosphere at 37°C overnight. The culture with the addition of hydrocortisone (10⁻⁷ M) was also used.

DNA extraction. Cell suspensions (2 × 10⁷/ml) of the thymus, bone marrow, spleen, and lymph nodes were washed once by centrifugation at 250 × g for 10 min. The cells were suspended in 1 ml of 50 mM Tris-HCl buffer at pH 8.0 containing 10 mM EDTA, 0.5% (wt/vol) sodium lauryl sarcosinate and 0.5 mg of proteinase K per ml and subsequently incubated for 1 h at 50°C. The lysates were micro-centrifuged for 14,000 × g for 5 min, and the supernatants,
which mainly contained DNA with a low molecular weight, were obtained (8). DNA in the supernatant was extracted twice with phenol-chloroform and once with chloroform and then precipitated by the addition of ethanol containing 0.2% MgCl₂ and 3 M sodium acetate at pH 5.2 to a concentration of 67% and kept overnight at −20°C. Finally, DNA was pelleted by centrifugation at 14,000 × g for 10 min and dried.

**DNA electrophoresis.** DNA was appropriately diluted with a solution containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0) to obtain the DNA recovered from a designated initial number of cells in a final volume of 5 µl. To eliminate overlapping bands of RNA, each DNA sample was treated for 10 min at 37°C with 10 µg of RNase that had been preheated to 100°C for 15 min. The samples were mixed with 2 µl of loading buffer, which contained 0.25% bromophenol blue and 40% sucrose in water, and heated at 65°C for 10 min. Electrophoresis was performed for 3 h at 40 V with 2% agarose gels containing ethidium bromide at a final concentration of 0.5 mg/ml in 40 mM Tris-40 mM acetic acid–1 mM EDTA (pH 8.0). DNA banding was evidenced by UV transillumination.

**DNA labeling technique and flow cytometric analysis.** The method for labeling was originally described by Nicoletti et al. (14). Briefly, the pellet of thymocytes was gently resuspended with 1 ml of hypotonic fluorochrome solution (50 µg of propidium iodide per ml in 0.1% sodium citrate plus 0.1% Triton X-100) in polypropylene tubes. The tubes were placed at 4°C in the dark overnight before flow cytometric analysis. The red fluorescence of individual nuclei was measured with a FACS-400 flow cytometer (Becton Dickinson, Mountain View, Calif.).

**Adrenalectomy.** Five-week-old BALB/c mice were surgically adrenalectomized under diethyl ether anesthesia. Mice which underwent surgery were injected subcutaneously with LPS (50 µg) 4 days after surgery and during this period were kept in a dark and quiet air-conditioned room.

**RESULTS**

**Induction of DNA fragmentation in the thymus by in vivo administration of LPS.** Whether the administration of LPS to mice induced apoptosis in the thymus was tested. Mice were injected i.p. with various doses of LPS from *E. coli* O111, and the appearance of fragmented DNA was examined by gel electrophoresis. The agarose gels viewed under UV light after ethidium bromide staining to visualize DNA are shown in Fig. 1. There was a roughly dose-dependent increase in fragmented DNA by injection with more than 10 µg of LPS. Maximal DNA fragmentation was achieved with mice injected with 100 µg of LPS, whereas no significant fragmented DNA was detected in those injected with 1 µg of LPS. Similar DNA fragmentation was observed in the thymus of mice injected with LPS from *S. enteritidis* or *K. pneumoniae* O3. In addition, there was no detectable DNA fragmentation in the thymus of 15-week-old mice by injection with 100 µg of any LPS.

**Time course of DNA fragmentation in the thymus of mice injected with LPS.** The time course of the appearance of fragmented DNA in the thymus of mice injected with 50 µg of LPS was monitored. The experimental results are shown in Fig. 2. Fragmented DNA was undetectable in the thymus 1 h after injection. Definite DNA fragmentation appeared 6 h after injection and reached the maximum 18 h after injection. DNA fragmentation lasted for 2 days, but little DNA fragmentation was detectable 4 days after injection. In addition, the cell numbers of thymocytes recovered at 18, 48, and 72 h after injection of LPS were ca. 50, 15, and 12% of that of normal thymus, respectively, indicating the approximate amount of apoptosis in the thymus.

**Detection of DNA fragmentation in thymocytes by laser flow cytometry.** We tried to confirm DNA fragmentation in the thymus by the laser flow cytometric analysis. Typical experimental results are shown in Fig. 3. The fluorescence-activated cell sorter histogram analysis revealed that the peak fluorescence of a portion of propidium iodide-stained thymocyte nuclei which were prepared from mice injected with 50 µg of LPS 16 h before being tested significantly shifted to the left, suggesting an increase of nuclei with subdiploid DNA content and a decrease of nuclei with diploid DNA content.

**Detection of DNA fragmentation in various lymphoid organs.** Whether apoptosis could be induced in other lymphoid organs as well as the thymus was investigated. The experimental results are shown in Fig. 4. There was no detectable DNA fragmentation in the bone marrow, spleen, or inguinal lymph nodes of mice injected with 50 µg of LPS 18 h after injection, although fragmented DNA was found in the thymus.

**Effect of LPS on cultured thymocytes in induction of apoptosis.** To test the possibility that LPS can directly cause apoptosis in thymocytes, thymocytes were cultured in vitro with LPS. The experimental results are shown in Fig. 5. The in vitro cocultivation of thymocytes with LPS (50 µg/ml) for 6 h did not cause DNA fragmentation. In contrast, the addition of hydrocortisone to the culture of thymocytes clearly induced it. There was no fragmented DNA in the culture of thymocytes at a LPS concentration of 100 µg/ml for 16 h (data not shown).

**Effect of adrenalectomy on induction of apoptosis in the thymus of mice injected with LPS.** Previously it was reported that the thymus was injured by corticosteroids (3, 4). In order to clarify the role of the adrenal hormone on apoptosis of the thymocytes by LPS, how adrenalectomy affects apoptosis in mice injected with LPS was determined (Fig. 6). The subcutaneous administration of LPS (50 µg) to the adrenalectomized mice did not cause a significant amount of
FIG. 2. Time course of apoptosis in the thymus of mice injected with LPS. Two mice per each experimental group were injected i.p. with 50 µg of LPS, and the appearance of DNA fragmentation in the thymus was examined at various hours (A) or various days (B) after injection. (A) Fragmented DNA from the thymus at 48 (lane 2), 18 (lane 3), 6 (lane 4), and 1 (lane 5) after injection and that of the untreated control (lane 1). (B) Fragmented DNA in the thymus at 4 (lane 3) or 2 (lane 2) days after injection and in the untreated control (lane 1). Molecular size markers, in kilobases, are on the left.

Fragmented DNA, although definite DNA fragmentation caused by injection with LPS was detectable in mice which had not undergone operations and those which had undergone sham operations.

**Participation of TNF-α in induction of apoptosis in the thymus of mice injected with LPS.** It is well known that TNF-α is released in mice injected with LPS and, further, that it leads various types of cells to apoptosis. Therefore, the possibility that TNF-α might play a role in induction of apoptosis in the thymus of mice injected with LPS was raised. Whether the administration of anti-TNF-α antibody could affect the appearance of apoptosis in the thymus of mice injected with LPS was investigated (Fig. 7). The

![Fluorescence Intensity vs. Cell Number](image1)

**FIG. 3.** Flow cytometric profile of DNA fluorescence of propidium iodide-stained thymocytes. Mice were injected i.p. with 50 µg of LPS 16 h before being tested. The histograms of untreated control mice (dotted line) and LPS-treated mice (solid line) are shown. The fluorescence intensity is expressed on a linear scale. The experimental data for one of three experiments are shown.

![DNA Fragmentation](image2)

**FIG. 4.** Induction of apoptosis in various lymphoid organs of mice injected with LPS. DNA fragmentation was estimated for the thymus (lanes 1 and 5), spleen (lanes 2 and 6), bone marrow (lanes 3 and 7), and lymph nodes (lanes 4 and 8) from untreated control mice (lanes 1 to 4) or mice injected i.p. with 50 µg of LPS (lanes 5 to 8) 18 h before being tested. Two mice per experimental group were used, and fragmented DNA was analyzed by gel electrophoresis. Molecular size markers, in kilobases, are on the left.
simultaneous i.p. administration of anti-TNF-α antibody (50 μg) partially inhibited the appearance of fragmented DNA in mice injected i.p. with LPS (50 μg). Partial inhibition was found in all of three experiments, although this inhibition was undetected in experiments with an irrelevant rat antiserum.

**DISCUSSION**

In the present study, we demonstrated that the administration of LPS to mice induced DNA fragmentation in the thymus. DNA fragmentation was confirmed by the gel electrophoresis and laser flow cytometric analysis. Because DNA fragmentation is one of the characteristics typical in apoptotic cell death (4, 9, 20), it was suggested that the cell death in the thymus of mice injected with LPS might be due to apoptosis. Previously, Rowlands et al. (15) and Baroni et al. (2) reported massive necrosis of thymic lymphocytes in mice injected with LPS. However, apoptosis was not fully clarified at that time, and they did not show evidence supporting necrotic cell death. Therefore, fragmented DNA observed in the present study clearly supported the apoptotic death of thymic lymphocytes. This report is the first description of apoptotic death of thymic lymphocytes in mice injected with LPS.

It seemed that immature thymocytes were sensitive to apoptosis caused by injection of LPS. This was supported by the finding that DNA fragmentation was predominantly detectable in the thymus but not in the lymph nodes or the spleen, because mature T cells were present mainly in the lymph nodes and the spleen. It was also supported by the fact that the injection of LPS into old mice did not cause DNA fragmentation in the thymus. Furthermore, we have already reported that small lymphocytes in the cortex disappeared 2 or 3 days after injection of LPS and that the cortex, in which immature cells are present, became thin (22, 23). Collectively, these results suggest that it is likely that immature thymocytes in the cortex are destroyed by apoptosis after injection of LPS.

Apoptosis in the thymus of mice injected with LPS may be mediated mainly by adrenal hormones, because the adrenalectomy completely inhibited the appearance of apoptosis in mice. There are several reports that the depletion of thymocytes after the injection of LPS may be caused by glucocorticoid hormones (6, 7, 16). It is, therefore, very likely that this apoptotic death of thymocytes is mainly due to the adrenal hormone released by the administration of LPS. On the other hand, anti-TNF-α antibody partially inhibited the apoptosis of thymocytes by LPS, suggesting the possibility that TNF-α participates in induction of apoptosis. However, we have no evidence that TNF-α directly induces apoptosis in the thymus by LPS because there was no significant
fragmented DNA in adrenalectomized mice caused by injection of LPS. The role of TNF-α in induction of apoptotic cell death in thymocytes is still unclear. However, it has been reported that TNF-α affects the sensitivity of the lymphocytes to the glucocorticoid hormone (3) and, further, that it regulates the synthesis of adrenal hormones (10, 11, 18). Therefore, the exact relationship between adrenal hormones and TNF-α in apoptosis induced by LPS remains to be clarified.

Cell death, which can proceed via apoptosis or necrosis, is now recognized to be of fundamental importance in many fields of biology. At present, the immune system provides good models for apoptosis, and most studies have been performed with in vitro experimental systems using cultured lymphocytes. Irradiation (17) and corticoids (5) are known to be typically used in induction of DNA fragmentation for apoptosis in in vivo experimental systems. In the present study, we demonstrated that LPS could provide a new experimental system for the study of in vivo apoptosis. Since apoptosis of thymocytes after injection of LPS is massively and uniformly induced in this particular cell type, it is suitable for the study of the molecular mechanism of apoptosis.

Here, we demonstrated that LPS, bacterial endotoxin, induced in vivo apoptosis in target cells, although induction was not the direct action of LPS. This is of particular interest because bacterial toxins, such as protein toxins, usually cause the death in target cells via necrosis.

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REFERENCES