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Lipopolysaccharide-Induced Apoptosis in Swine Lymphocytes In Vivo

MARI NORIMATSU,[1]* TOMOKO ONO,[1] AKEMI AOKI,[1] KOHJI OHISHI,[1] TOSHIO TAKAHASHI,[1] GEN WATANABE,[2] KAZUYOSHI TAYA,[2] SHUJI SASAMOTO,[2] AND YUTAKA TAMURA[1]

National Veterinary Assay Laboratory, Kokubunji, Tokyo 185,[1] and Laboratory of Veterinary Physiology, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183,[2] Japan

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The in vivo effects of bacterial lipopolysaccharide (LPS) on the immune systems of piglets were investigated. Intravenous injection of 0.5 mg of LPS per kilogram of body weight induced apoptosis, which was characterized by nuclear chromatin condensation and fragmentation and a ladder formation of nucleosomal DNA in lymphocytes both in the cortex of the thymus and in the germinal centers and paracortical areas of mesenteric lymph nodes at 24 h postinjection. The levels of endotoxin, tumor necrosis factor alpha, and cortisol in serum increased, generally according to the dose of LPS. These findings suggest that LPS can induce in vivo apoptosis of lymphocytes in piglets and support the notion that cytokine and endocrine responses may play an important role in LPS-induced apoptosis in the immune system.

In domestic pigs, chronic infections, which bring about reduction of feed efficiency, elevation of prophylactic medication and vaccination costs, and animal death, have recently been attracting more attention than acute epidemic infections. These chronic infections are mainly caused by gram-negative bacteria. Lipopolysaccharides (LPS), which are common components of the cell walls of gram-negative bacteria, are capable of eliciting a wide variety of pathophysiological effects, such as endotoxin shock, tissue injury, and lethality, in both humans and animals (17, 18). Inoculations of some domestic animals with gram-negative whole-cell vaccines are now increasing, on the basis of epidemiological backgrounds. Accordingly, it seems important to clarify the effects of LPS not only on immunogenicity but also on toxicity in domestic animals so that a safety assessment of such vaccines can be made.

LPS is known to be a potent stimulant of the host immune system (14, 16). There are many investigations that have concluded that LPS is a mitogen that stimulates the proliferation and differentiation of B cells and initiates an activation of macrophages, resulting in an enhancement of immune responses (11, 23, 26). However, little is known about the effects of LPS on the thymus or T cells in vivo, with the exception of several reports on thymocyte depletion in mice injected with LPS (3, 31, 32, 34).

In this study, we investigated the in vivo effects of LPS on the immune system of piglets and focused also on the cytokine-endocrine system, which has recently been considered to be important in inflammatory responses (22, 25).

LPS extracted from *Escherichia coli* O55:B5 by the Westphal method was purchased from Difco Laboratories (Detroit, Mich.). LPS was suspended at various concentrations in LPS-free saline (Otsuka, Tokyo, Japan).

Six male and seven female 2-month-old, specific-pathogen-free piglets (body weight, 20 to 30 kg each) were purchased from Sasaki Farm Ltd. (Chiba, Japan). They were injected intravenously with various doses of LPS (see Table 1). Blood samples from piglets 1, 2, 4, 7, 10, 12, and 13 were collected from the cervical vein at 0, 1, 3, 6, 9, 24, 48 and 120 h postinjection (hpi), and each serum sample was stored at −80°C until used. For peripheral blood cell counts, leukocytes were diluted with Türk solution and then counted with a hemocytometer. One animal injected with LPS-free saline served as a control. All the animals, with the exception of one piglet which had died, were sacrificed by euthanasia at 24 or 120 hpi and were immediately processed for histopathological examination. The piglet which died was necropsied immediately.

For light-microscopic examination, the cerebrum, cerebellum, thymus, heart, lungs, intestines, spleen, mesenteric lymph nodes, pancreas, kidneys, and adrenal glands were fixed in 10% neutral buffered formalin. Paraffin sections (thickness, 2 μm each) were made and stained with hematoxylin and eosin. For electron-microscopic examination, small pieces of the formalin-fixed tissues were postfixed in 1.0% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) and embedded in epoxy resin (Quetol 812; Nisshin EM Co. Ltd., Tokyo, Japan). Ultrathin sections were double stained with uranyl acetate and lead citrate and observed with a JEM-100S electron microscope (JEOL Ltd., Tokyo, Japan) at 80 kV.

TABLE 1. Clinical and hematological findings in LPS-injected piglets

Piglet	Dose (mg/kg)	Temperature (°C)[a]	Leukocytes (cells/mm³)		Vomiting[b]	Ananastasia[c]
			0 hpi	Minimum no.		
1[d]	2	41.6	22,000	200	+	+
2	0.5	41.3	17,800	400	+	++
3	0.5	41.5	16,200	600	+	++
4	0.0625	41.0	12,000	2,100	+	+
5	0.0625	41.4	15,100	400	+	+
6	0.0625	40.5	14,300	4,800	+	+
7	0.0078	41.3	16,100	400	+	±
8	0.0078	41.3	22,200	1,000	+	±
9	0.0078	41.0	10,500	6,000	−	−
10	0.001	41.1	15,000	1,000	−	−
11	0.001	40.8	5,500	5,500	−	−
12	0.0001	40.1	10,700	9,900	−	−
13	0	40.1	19,900	19,900	−	−

[a] Maximum body temperature during observation period.
[b] +, observed; −, not observed.
[c] −, not detected; ±, transiently observed; +, observed for about half a day; ++, observed for more than 1 day.
[d] Died within 24 hpi.

* Corresponding author. Mailing address: National Veterinary Assay Laboratory, 1-15-1, Tokura, Kokubunji, Tokyo 185, Japan. Phone: 81-423-21-1841. Fax: 81-423-21-1769.

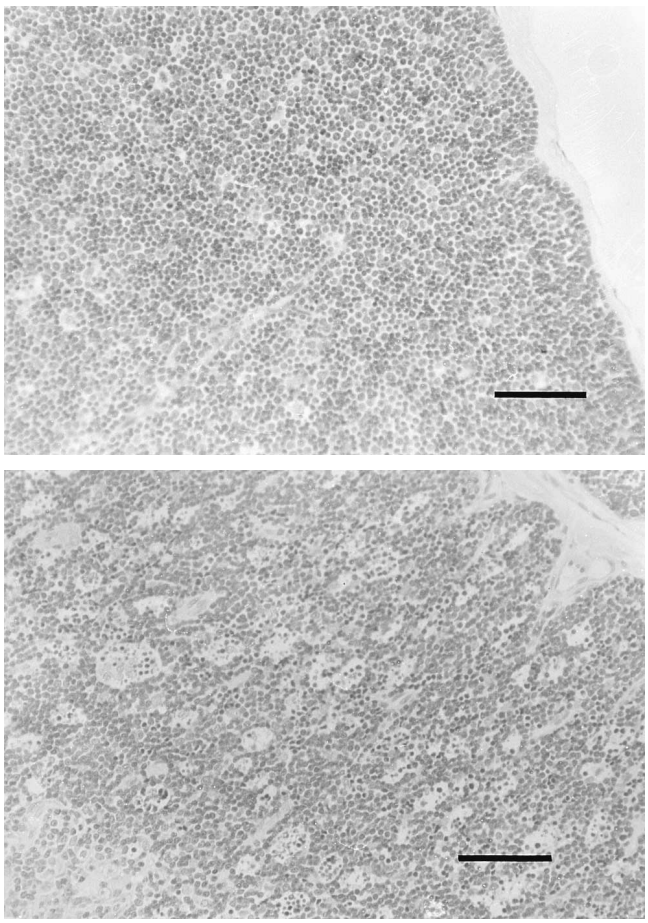

FIG. 1. Light microscopy of the thymus cortex of a control piglet (top) and an LPS-injected (0.5 mg/kg) piglet (bottom) at 24 hpi. Hematoxylin and eosin stain was used. (Top) Normal structure; (bottom) multiple foci of cortical lymphocytes showing pyknosis or karyorrhexis. Bars, 60 μm.

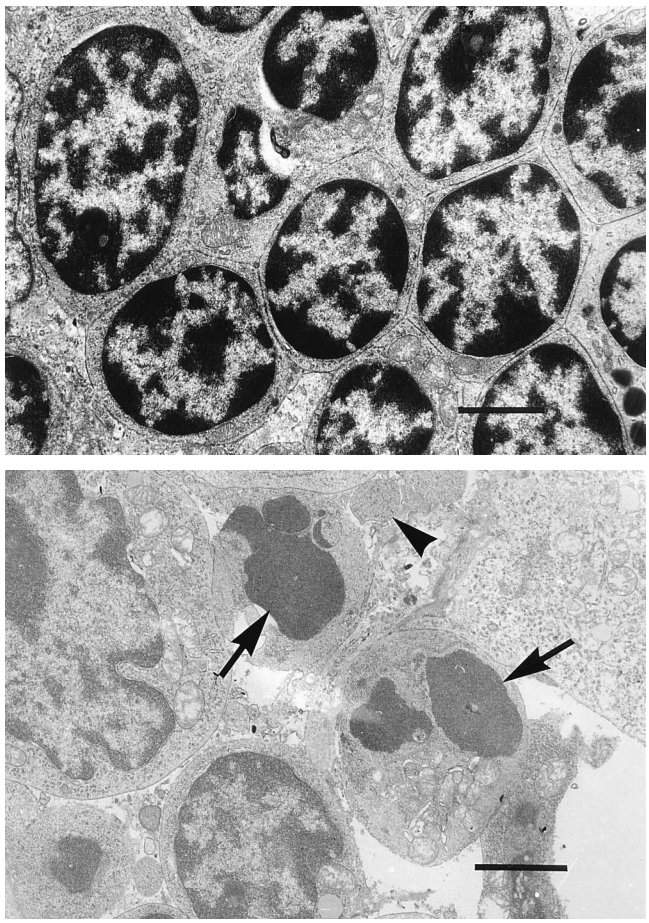

FIG. 2. Electron microscopy of the thymus cortex of a control piglet (top) and an LPS-injected (0.5 mg/kg) piglet (bottom) at 24 hpi. (Top) Normal structure of thymocytes; (bottom) marked condensation of nuclear chromatin (arrows) and apoptotic bodies (arrowhead) of thymocytes. Bars, 2 μm.

At 24 hpi, lymphocytes were separated from freshly isolated thymus, spleen, and mesenteric lymph node tissues with wire mesh, collected in 0.1 M phosphate-buffered saline, and washed three times. DNAs from these cells were then extracted with the DNA extractor WB kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The extracted DNAs were mixed with TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and subjected to electrophoresis in a 2% agarose gel. The gel was stained with ethidium bromide at 1 μg/ml and visualized with UV light. For precise examination, the stained gel was observed with a FluorImager (Molecular Dynamics Co. Ltd., Sunnyvale, Calif.).

The endotoxin levels in the serum were assayed with the *Limulus* J test Wako (Wako Pure Chemical Industries, Ltd.). Briefly, endotoxin-free glasswares were prepared by heating them in an oven at 250°C for 2 h. The serum samples were mixed with 9× Sample Pretreatment Solution (Wako Pure Chemical Industries, Ltd.), which contains Triton X-100, and incubated at 80°C for 5 min. Then equal amounts of *Limulus* amoebocyte lysate were added to each sample, and the samples were incubated at 37°C. The gelation time was measured with a Toxinometer ET-201 (Wako Pure Chemical Industries, Ltd.), and the concentration was calculated with an LS Toximaster (Wako Pure Chemical Industries, Ltd.).

Tumor necrosis factor alpha (TNF-α) levels in serum were measured by the tetrazorium salt cytotoxicity assay with the murine fibrosarcoma cell line WEHI 164, clone 28 (10). The specificity of the TNF-α was confirmed by the inhibition or reduction of the activity by preincubation of the samples with rabbit anti-mouse TNF-α antibody (Genzyme, Cambridge, Mass.). The detection limit of the TNF-α in the serum was 10 pg/ml.

Concentrations of cortisol in serum were determined by a double antibody radioimmunoassay system with a ^{125}I-labelled radioligand. The labelled steroid was cortisol 3-(*O*-carboxymethyl)oximino-(2-[^{125}I]iodohistamine) (74 TBq/mmol), which was purchased from Amersham International plc. The antiserum against cortisol (HAC-AA71-02RBP85) was provided by K. Wakabayashi (Gunma University, Maebashi, Japan). The method used for the determination of cortisol has been described previously (28). The intra- and interassay coefficients of variation were 7.7 and 16.7%, respectively.

As shown by the results in Table 1, piglets injected with more than 0.001 mg of LPS per kg of body weight became highly feverish (>41°C) within 24 hpi. Among them, piglets injected with 2 or 0.001 mg of LPS per kg showed an endotoxic reaction-characteristic fever, which peaked at about 1 and 3 hpi. Piglets injected with more than 0.001 mg of LPS per kg also showed a transient leukopenia which peaked at 3 hpi and then recovered by 24 hpi. Piglets injected with more than 0.0078 mg

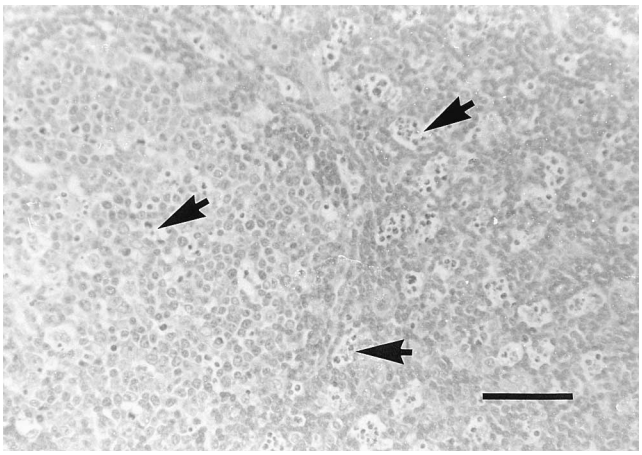

FIG. 3. Light microscopy of a mesenteric lymph node of an LPS-injected (0.5 mg/kg) piglet at 24 hpi. Small foci of follicular and/or paracortical lymphocytes showing pyknosis or karyorrhexis (arrows) are shown. Hematoxylin and eosin stain was used. Bar, 30 μm.

of LPS per kg showed vomiting within 1 hpi and then developed ananastasia. Piglets injected with 0.5 mg of LPS per kg showed an inability to support themselves on their hind limbs at 24 hpi, and the piglet injected with 2 mg of LPS per kg died at 18 hpi. The piglet injected with 0.0001 mg of LPS per kg and the control animal showed no clinical signs.

Histopathological changes developed at 24 hpi (at 18 hpi in the piglet injected with 2 mg of LPS per kg), and the severity of the changes was generally proportional to the injected dose of LPS. In the thymuses of piglets injected with more than 0.5 mg of LPS per kg, small foci of cortical lymphocytes showing karyorrhexis or pyknosis were frequently observed (Fig. 1). By electron-microscopic examination, cortical lymphocytes showed marked nuclear chromatin condensation and fragmentation by comparison with those of the control (Fig. 2, top), and some of these cells formed crescent-shaped chromatin condensation and/or apoptotic bodies (Fig. 2, bottom). Changes similar to those in the thymus were observed in the paracortical areas and germinal centers of mesenteric lymph nodes (Fig. 3). In the spleen, these changes in the lymphocytes were observed in the lymphoid follicles in the dead piglet but only the depletion of lymphocytes in the lymphoid follicles was observed in the sacrificed animals. As to the changes in other organs, prominent congestion was observed in the pyloric mucosa of the stomachs of piglets injected with more than 0.5 mg of LPS per kg. Thrombosis in glomerular capillaries and degeneration of tubular epithelial cells were observed in the kidney of the piglet injected with 2 mg of LPS per kg. Thrombosis was found in the tela chorioidea of the oblongata of the piglet injected with 0.5 mg of LPS per kg, and it also developed hepatocellular necrosis with mononuclear cell infiltration in the liver. The above-mentioned lesions, however, disappeared at 120 hpi.

As shown in Fig. 4, DNAs extracted from the thymus and mesentreric lymph nodes of the piglet injected with 0.5 mg of LPS per kg showed a ladder formation of multiples of about 200 bp at 24 hpi, indicating a fragmentation of nucleosomal DNA by endonuclease. DNAs extracted from the spleen of LPS-injected piglets and from the above-mentioned lymphoid organs of the control showed no ladder formation of multiples.

As shown in Fig. 5, endotoxin in serum was detected dose dependently at 1 hpi in all piglets injected with LPS. After that, the endotoxin levels gradually decreased and disappeared by 3 to 120 hpi. In piglets which were injected with 0.5 mg of LPS

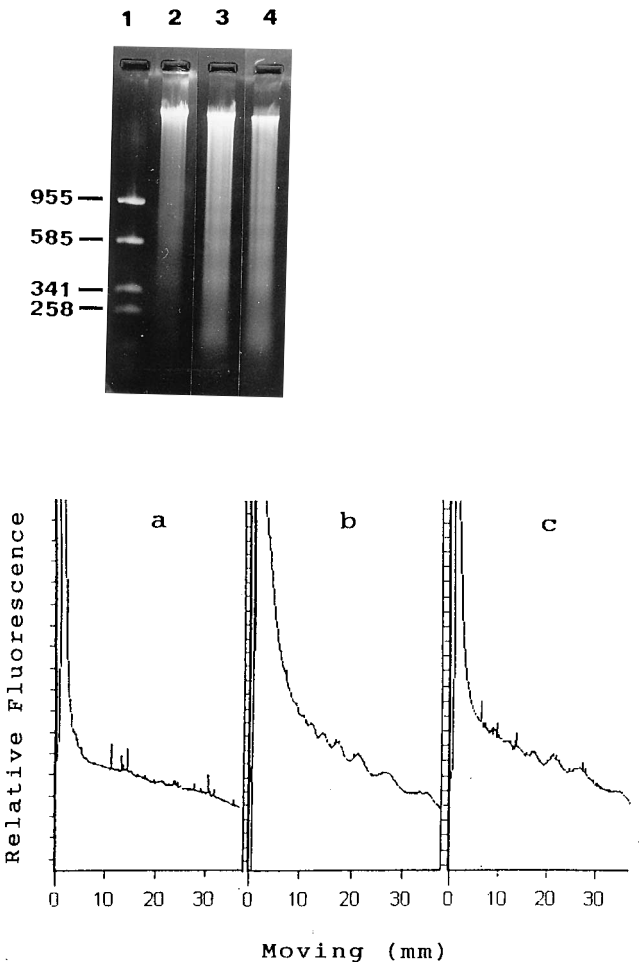

FIG. 4. DNA fragmentation in the thymus and mesenteric lymph node. (Top) Lanes: 1, molecular size standards; 2, thymus cells from the control animal; 3, thymus cells from an LPS-injected piglet at 24 hpi; 4, mesenteric lymph node cells from an LPS-injected piglet at 24 hpi. The numbers at the left are base pairs. (Bottom) Analysis by FluorImager. (a) Thymus cells from a control animal. (b) Thymus cells from an LPS-injected piglet at 24 h. (c) Mesenteric lymph node cells from an LPS-injected piglet at 24 hpi.

per kg and which showed severe clinical signs after 24 hpi, endotoxin levels were relatively high at 24 hpi.

As shown in Fig. 6, most of the LPS-injected piglets showed marked elevations in TNF-α activity only at 1 hpi. However, in the piglet injected with 2 mg of LPS per kg, the elevation in TNF-α activity continued to 6 hpi. The elevation in TNF-α activity was dependent on the dose of LPS and was not detected in the piglets which showed no clinical signs. The elevation in TNF-α activity was dependent on the dose of LPS and was not detected in the piglets which showed no clinical signs. The elevations in TNF-α activity were inhibited or reduced by anti-mouse TNF-α rabbit antibody.

As shown in Fig. 7, serum cortisol levels peaked at 1 to 3 hpi and recovered to normal by 9 hpi, except for the piglet injected with 2 mg of LPS per kg. In the piglets which showed no clinical signs, there was no clear increase in cortisol levels. In addition, the changes in cortisol levels due to circadian rhythm and bleeding stress in piglets were within 20 ± 15 ng/ml.

In this study, we demonstrated that bacterial LPS could induce a depletion of lymphocytes in the lymphoid organs of 2-month-old, specific-pathogen-free piglets. Electron-micro-

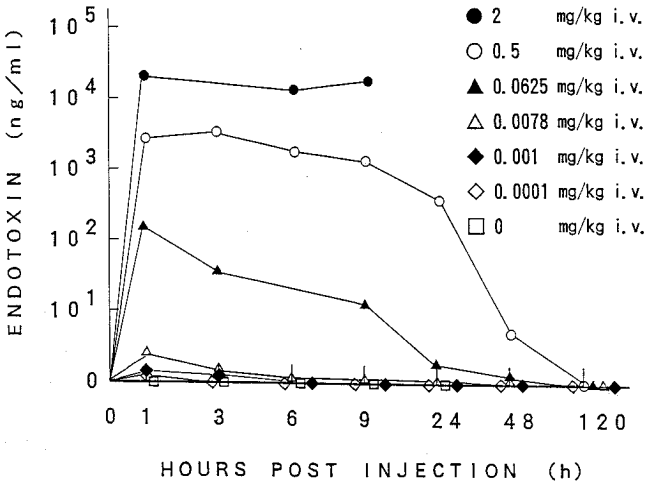

FIG. 5. Endotoxin levels in the serum of LPS-injected piglets. i.v., intravenous.

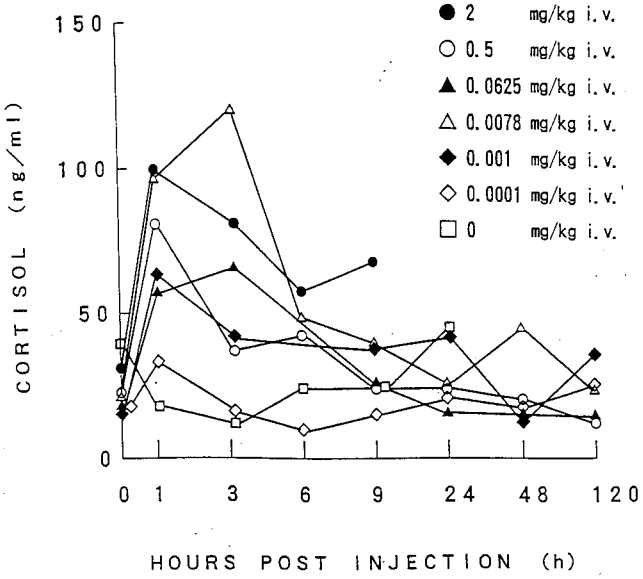

FIG. 7. Cortisol levels in the serum of LPS-injected piglets. i.v., intravenous.

scopic and electrophoretic examinations of piglets injected with 0.5 mg of LPS per kg revealed that this lymphocyte depletion was brought about by apoptosis in the thymus and mesenteric lymph nodes. In the spleen, lymphocyte apoptosis may occur earlier, because karyorrhexis or pyknosis suggesting apoptosis was detected with a light microscope in the spleen of the piglet which was injected with 2 mg of LPS per kg and died at 18 hpi. As for this dead piglet, we believe that lymphocyte apoptosis occurred in the thymus, mesenteric lymph nodes, and spleen because karyorrhexis or pyknosis was observed with a light microscope. The present study seems to be the first report of in vivo lymphocytic apoptosis in pigs due to LPS. In this connection, Stine et al. (27) reported the detection with a light microscope of changes in lymphocytes in mice and pigs injected with live *Actinobacillus pleuropneumoniae* that were similar to those reported in this study, but they did not characterize those changes in the lymphocytes as apoptosis.

In addition to lymphocytic apoptosis, we observed pathological changes such as intravascular coagulation in some organs and centrilobular necrosis of hepatocytes in the liver. The incidence and severity of these histopathological changes were generally proportional to the dose of LPS. These coagulative and degenerative changes are known as endotoxin-induced lesions; however, the relationship between these pathological changes and lymphocyte apoptosis remains to be seen.

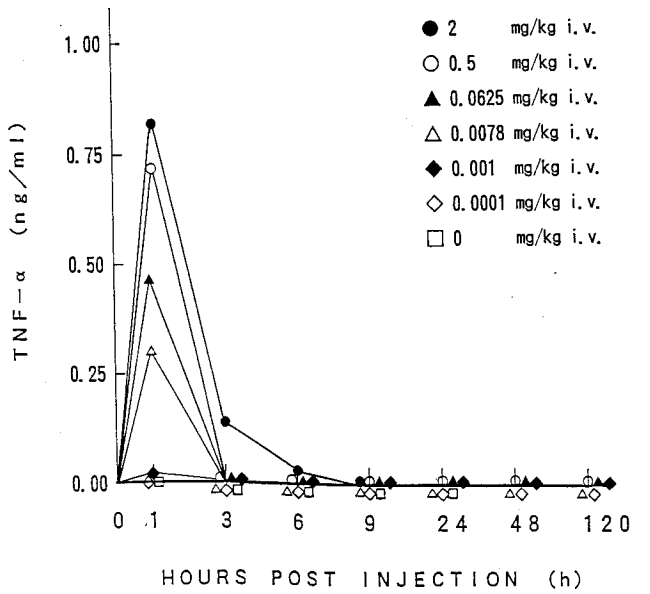

FIG. 6. TNF-α levels in the serum of LPS-injected piglets. i.v., intravenous.

It has been well known that immature T cells and some T cell hybridomas (1, 15) develop apoptosis after exposure to several substances, including LPS (34), whereas there are few reports of apoptosis in more mature lymphocytes in vivo, i.e., lymphocytes in the spleen or lymph nodes. Recently, we detected LPS-induced apoptosis in lymphocytes not only in the thymuses but also in the spleens and mesenteric lymph nodes of LPS-sensitive C3H/HeN mice (21). In this strain of mice, apoptotic changes were more severe in the thymus than in the spleen and lymph nodes after more than 1 mg of LPS per kg was injected. In the present study, the apoptotic changes detected in the thymus and mesenteric lymph nodes of piglets were less severe than those detected in C3H/HeN mice and there was no apparent difference in the severities of apoptotic changes in the thymus and mesenteric lymph nodes. The difference between piglets and mice in the susceptibilities of their lymphocytes to apoptosis may be caused by differences in immunological and endocrinological responses to LPS and/or in sexual maturation, because the lethal effects of LPS are similar between these two species (the minimum lethal dose was 2 mg/kg for piglets, and the 50% lethal dose was 3 mg/kg for C3H/HeN mice [21]). In addition, the susceptibilities of their lymphocytes to LPS and/or other mediators may be different between these two species.

Recently, an increasing number of examinations have revealed the existence of a network composed of cytokine, nerve, and endocrine systems. In this network, early inflammatory cytokines such as TNF-α and interleukin 1 activate the release of glucocorticoids through the pituitary glands (25). Glucocorticoids then inhibit the production of TNF-α (5, 9, 24, 29) and interleukin 1 (4, 12, 13, 19) and lead to a recovery from inflammation. The protective effects of glucocorticoids against lethality and cytokine responses have been investigated in hu-

mans and animals (2, 8, 35–37). On the other hand, corticosteroids are also known to suppress several functional capabilities of the immune system (6, 20) and have been reported to induce apoptosis of thymocytes (30, 33). The present data in piglets which showed no clinical signs were that TNF-α activity levels were below the lower limit of the detectable level and that serum cortisol levels did not differ from that of the control, though endotoxin was detected in the serum samples of the piglets which showed no clinical signs. Therefore, our data support the above-mentioned cytokine-nerve-endocrine network theory.

Apoptosis also plays an important role in the immune system (1, 7). It is believed that a depletion of lymphocytes in the lymphoid organs after an infection and/or an injection of gram-negative whole-cell vaccine may influence the prognosis by altering immunological functions. Further studies should be conducted to elucidate a precise mechanism of in vivo lymphocytic apoptosis and immunomodulation induced by LPS.

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