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Immobilization Stress Increases Hepatic IL-6 Expression in Mice

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When mice were subjected to restriction of movement in a small cylinder (immobilization stress), the serum interleukin (IL)-6 level rose in 1 h, following increased expression of IL-6 mRNA in both the liver and the spleen. The IL-6 mRNA induction was much greater in the liver than in the spleen when compared on a whole-organ basis. Intraperitoneal injection of bacterial lipopolysaccharide (LPS) also increased IL-6 mRNA expression in these organs, but more preferentially in the spleen. Immunohistochemical examinations of liver tissue using an antibody against murine IL-6 revealed that immobilization stress induced IL-6 mainly in hepatic parenchymal cells, whereas LPS injection did so only in sinusoidal mononuclear cells. These results indicate that immobilization stress induces IL-6 production in the liver, especially in hepatic parenchymal cells, probably by a different mechanism from that for IL-6 induction by LPS.

Interleukin (IL)-6 acts as one of the pivotal cytokines in host defense responses to infection and inflammation by activating lymphocytes and inducing hepatic acute phase proteins (1). It has been demonstrated that the blood IL-6 level is increased after application of not only inflammatory stimuli but also physiological and psychological stressors such as electric shock and immobilization (2), suggesting that IL-6 also plays a significant role in stress responses. In fact, IL-6 is known to stimulate the secretion of adrenocorticotropic hormone (ACTH), a representative stress-related hormone (3).

The major source of blood IL-6 after inflammatory stimuli seems to be macrophages, because bacterial lipopolysaccharide (LPS) induces IL-6 mRNA expression in macrophage-rich organs such as the spleen, liver, and lung *in vivo* (4), and also stimulates macrophages to produce IL-6 *in vitro* (1). Recently, Takaki et al. (5) reported that the blood IL-6 response to immobilization stress in rats is little influenced by splenectomy but attenuated by partial hepatectomy. Moreover, we showed that intracranial injection of IL-1β mimics the blood IL-6 response to immobilization stress, inducing IL-6 mRNA expression in the liver and spleen (6). Although these previous results suggest stress-induced synthesis and secretion of IL-6 in the liver, there is no direct evidence for this idea. In the present study, we examined IL-6 mRNA expression in the mouse liver and spleen after immobilization. Immunohistochemical examinations of the liver were also carried out to identify the type of cells producing IL-6.

MATERIALS AND METHODS

Animals and treatments. Male C57BL/6 mice (7-8 wks. old, SLC, Shizuoka, Japan) were housed in a plastic cage at 24 ± 1°C with a 12-h light-dark cycle (lights on at 7:00 h-19:00 h) and given free access to laboratory chow and water. Immobilization of mice was performed by transferring them from their home cage to a small meshed cylinder (3 × 6 cm). After immobilization for 0, 0.5, 1, and 2 h, mice were sacrificed by cervical dislocation. Blood was collected from the right ventricle, and the liver and spleen were excised for RNA preparation and immunohistochemical examination. In another series of experiments, mice were injected intraperitoneally with 3 mg/kg LPS (*E.coli* 055:B5; Difco, Detroit, MI, USA), and before and 2 h after the injection, the liver and spleen were excised. All experiments were performed between 8:00 h and 11:00 h.

Assay of serum IL-6. The concentration of serum IL-6 was measured using the IL-6-dependent cell line MH60.BSF2 (a gift from Dr. T. Matsuda, Osaka University, Suita, Japan) as previously described (7). The minimum detectable concentration of serum IL-6 was 2.0×10^{-2} U/ml (4.0 pg/ml of recombinant human IL-6).

Northern blot analysis. Total RNA was extracted by the guanidine isothiocyanate method using TRIzol solution (Gibco BRL, Gaithersburg, MD, USA), according to the manufacturer's directions. Poly $(A)^+$ RNA was prepared using oligo-d(T) cellulose columns (Clontech, Palo Alto, CA, USA), denatured at 70°C, separated on 1% agarose/formaldehyde gel, and transferred to and fixed on a nylon membrane (Amersham, Buckinghamshire, UK). An IL-6

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cDNA probe corresponding to nucleotides 63 to 713 of the published cDNA sequence (8) was prepared by reverse transcription-polymerase chain reaction from total RNA extracted from rat spleen, and was labeled with (α-^{32}P) dCTP using a multiprime DNA labeling kit (Amersham). Nylon membranes were hybridized with the labeled cDNA probe, washed, and exposed to x-ray films. The membranes were also rehybridized with a human β-actin cDNA probe (Wako, Osaka, Japan) as a reference.

Immunohistochemistry. The tissues were frozen in liquid nitrogen with the aid of OCT embedding medium (Miles, Elkhart, IN, USA). Cryostat sections of the tissues (6 μm thick) were examined according to the avidin-biotin complex method using a commercial kit (Nichirei, Tokyo, Japan) and a rabbit anti-mouse-IL-6 polyclonal antibody (R&D system, Minneapolis, MN, USA). Inactivation of endogenous peroxidase was performed by treating the sections with 0.9% H_2O_2 in methanol. Non-immunized rabbit serum was used as a negative control. Sections were also counterstained with hematoxylin.

Data analysis. All values were expressed as means ± SE. Statistical comparison of serum IL-6 levels was made by analysis of variance, followed by Scheffe's F test.

RESULTS

To confirm the blood IL-6 response to immobilization stress reported previously (2), we measured the serum IL-6 level after immobilizing mice in a small cylinder. As shown in Fig. 1, the serum IL-6 level was lower than 0.1 U/ml before immobilization, but rose gradually after immobilization to reach about 1.4 U/ml at 2 h.

Since contributions of the spleen and liver have been proposed in the blood IL-6 responses to inflammatory stimuli and some types of stressors, the IL-6 mRNA level was measured in these organs by Northern blot analysis. We first tried to analyze the mRNA using up to 40 μg of total RNA. However, no signal was detected under our experimental conditions, probably because of low expression of IL-6 mRNA (data not shown). Next, 25 μg of poly (A)$^+$ RNA was purified from the total RNA and subjected to analysis. As shown in Fig. 2A, a weak but clear signal of IL-6 mRNA was detected in spleen, and its intensity was increased in 0.5-2 h after immobilization. In liver, IL-6 mRNA was also detected but only at 0.5 h after immobilization. Thus, immobilization increased IL-6 mRNA expression in both spleen and liver. The total amount of poly (A)$^+$ RNA recovered was about 80 μg from the whole liver, and about 4 μg from the whole spleen. When the total poly (A)$^+$ RNA was subjected to analysis, no signal of IL-6 mRNA was detected in spleen even after immobilization, whereas there was a strong signal in liver at 0.5 h after immobilization (Fig. 2B). The effects of intraperitoneal injection of LPS on the IL-6 mRNA level were also examined. Comparative expression of IL-6 mRNA was found 2 h after LPS injection in the liver and spleen when total poly (A)$^+$ RNA isolated from the whole organ was analyzed (Fig. 2B).

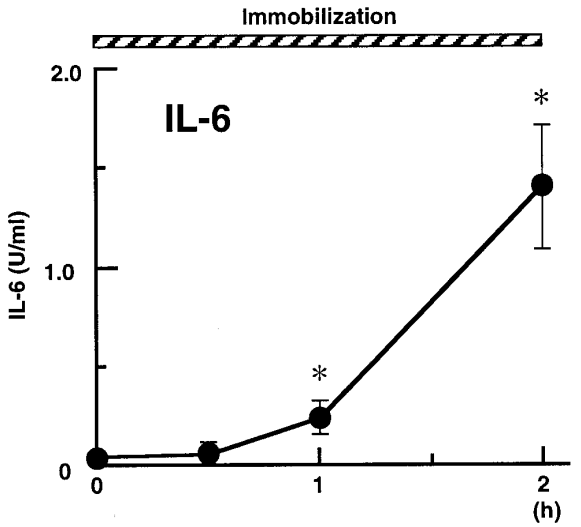

FIG. 1. Change in serum IL-6 concentration caused by immobilization stress. Mice were immobilized in a small cylinder, and blood was collected from the right ventricle. The serum IL-6 concentration was determined by the bioassay method using an IL-6-dependent cell line. Values are means ± SE for 9-11 mice. * $P < 0.05$ compared with 0 h.

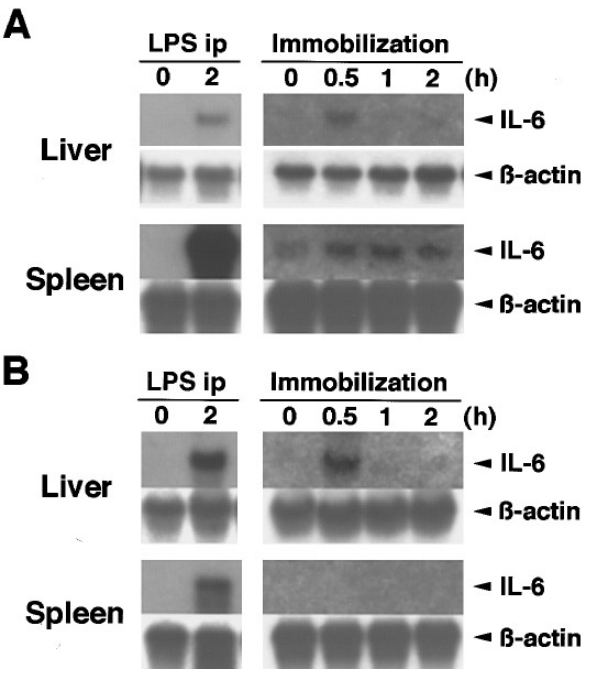

FIG. 2. Effect of immobilization stress or LPS injection on the hepatic and splenic IL-6 mRNA expression. Poly (A)$^+$ RNA was prepared from livers and spleens of mice subjected to immobilization or LPS-injection. (A) Poly (A)$^+$ RNA (LPS, 10 μg; immobilization, 25 μg) was used for Northern blot analysis. (B) Total poly (A)$^+$ RNAs from whole organ (liver 80 μg, spleen 4 μg) were used for the analysis. Results are representative of three independent experiments.

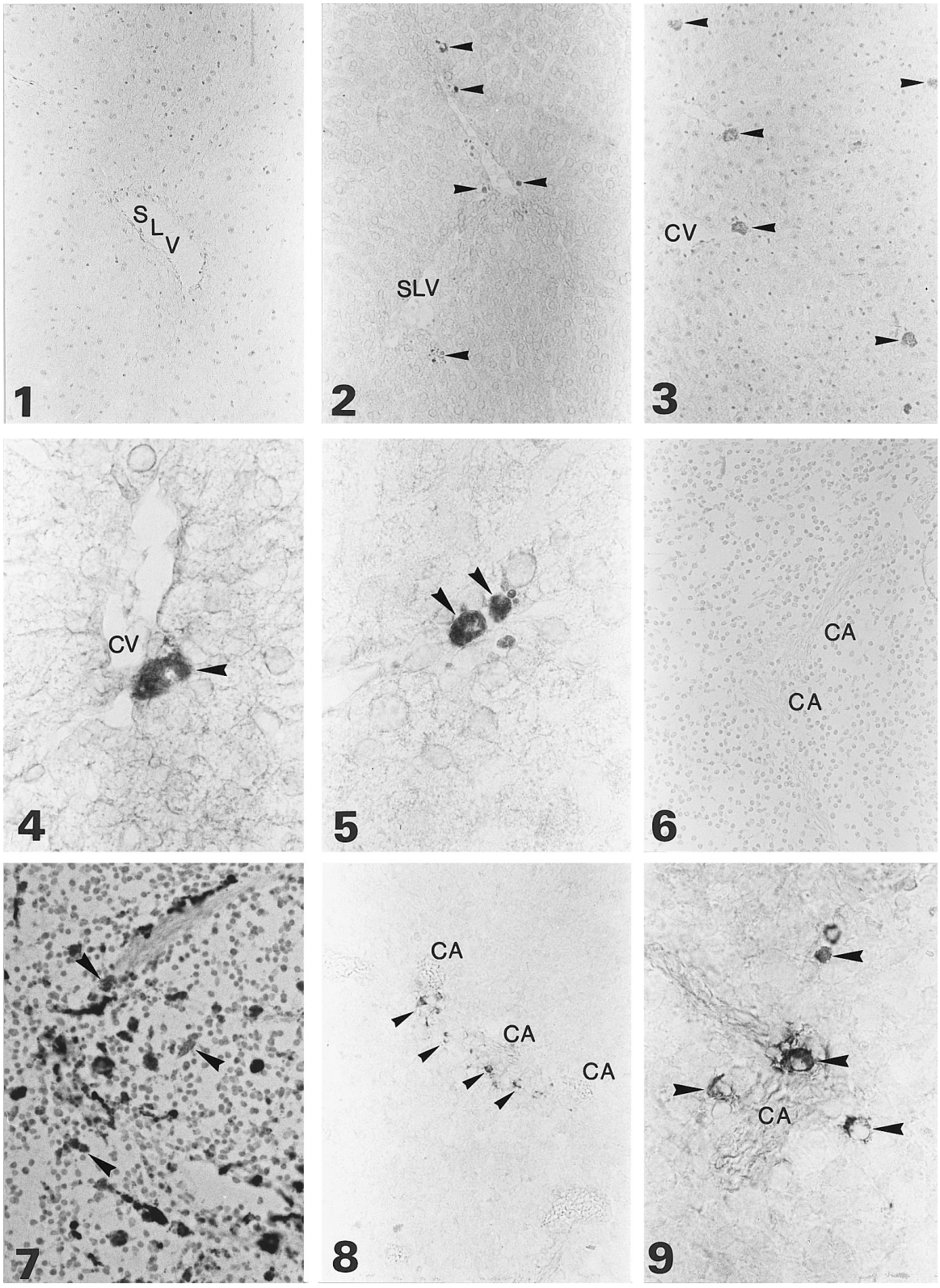

FIG. 3. Localization of IL-6 immunoreactivity in liver and spleen after immobilization or LPS injection. Mice were treated as in Fig. 2, and cryostat sections of liver (1-5) and spleen (6-9) were used for immunoperoxidase staining for IL-6. (1) Intact liver. ×130. (2) Liver at 2 h after LPS injection. Arrowheads show non-parenchymal cells positive for IL-6 around the sublobular vein (SLV). ×130. (3) Liver after 2-h immobilization. Arrowheads show parenchymal cells positive for IL-6. CV, central vein. ×130. (4) High magnification of part 3. An arrowhead shows a hepatocyte with strong IL-6 immunoreactivity. ×520. (5) High magnification of another field close to that of part 3. Arrowheads show mononuclear cells positive for IL-6. ×812.5. (6) Intact spleen. ×130. (7) Spleen at 2 h after ip injection of LPS. Many hemosiderin-containing macrophages are seen in the red pulp. Arrowheads show cells positive for IL-6. CA, central artery. ×160. (8) Spleen after 1-h immobilization. ×130. (9) High magnification of another field close to that of part 8. ×650. Results are representative of five independent experiments.

To identify the type of cells producing IL-6, tissue sections were examined immunohistochemically by using an anti-IL-6-antibody. In liver, IL-6-positive cells were undetected before and 0.5 h after immobilization (Fig. 3-1), but appeared abundantly 1 and 2 h after immobilization (Fig. 3-3). The IL-6 immunoreactivity was present mainly in hepatic parenchymal calls, and sparsely in sinusoidal cells (Figs. 3-3, -4 and -5). LPS treatment also induced IL-6 immunoreactivity in sinusoidal mononuclear cells but not in hepatic parenchymal cells (Fig. 3-2). In spleen, immobilization induced IL-6 immunoreactivity in the region of red pulp, and also around the white pulp arteries and central arteries (Figs. 3-6, -8, and -9). LPS injection also induced stronger IL-6 immunoreactivity in spleen, but only in the red pulp (Fig. 3-7). Some IL-6-positive mononuclear cells had hemosiderin-like blackish granules, suggesting they were macrophages.

DISCUSSION

The present studies demonstrated that immobilization stress induced the expression of IL-6 mRNA in both liver and spleen, followed by an increase in the serum IL-6 level. When the IL-6 mRNA responses of the two organs were compared on a whole-organ basis, the response of the liver was much larger because of the much greater content of total IL-6 mRNA, suggesting that the liver, more than the spleen, contributed to the immobilization-induced increase of the serum IL-6 level. This seems consistent with the report of Takaki et al. (5) that the blood IL-6 response to immobilization stress is attenuated by partial hepatectomy, but little influenced by splenectomy. The IL-6 mRNA induction by LPS administration was comparable in the spleen and liver. In addition, the LPS-induced increase in the serum IL-6 level was reported to be much depressed by splenectomy (4) These results suggested a significant contribution of the spleen to the blood IL-6 response to inflammatory stimuli.

The most interesting finding in the present study is that the major cells producing IL-6 in the liver were apparently different in inflammatory and non-inflammatory stress conditions: that is, LPS-injection induced IL-6 mainly in sinusoidal cells, probably Kupffer's cells. On the other hand, immobilization stress induced IL-6 mainly in hepatic parenchymal cells. It is well known that Kupffer's cells synthesize IL-6 in response to LPS. *In vitro* studies have also shown that hepatocytes are capable of producing IL-6 (9, 10). Our results are, as far as we know, the first demonstration of IL-6 synthesis in hepatic parenchymal cells *in vivo*.

As in the liver, IL-6 producing cells in the spleen seem rather different under inflammatory and non-inflammatory stress conditions: that is, IL-6 was induced in the region of red pulp under both conditions, but around the white pulp and central arteries after immobilization stress. Although the mechanism for these different patterns of IL-6 induction is unclear at present, one possible candidate is the participation of the catecholaminergic mechanism, which is activated by immobilization stress. It has been shown that many sympathetic nerve terminals are located along splenic arteries (11) and take part in the modulation of splenocyte activities, including IL-6 mRNA expression (6). A role for the sympathetic nervous system was also proposed in immobilization-induced IL-6 production in the liver (5).

There are several possible explanations for the biological significance of IL-6 production in the liver. Hepatic IL-6 may be a local stimulator of the hepatic synthesis of acute phase proteins. It is known that acute phase protein synthesis is increased by non-inflammatory stress as well as inflammation (12). It is thus likely that IL-6 produced from hepatic parenchymal cells may effectively activate acute phase protein synthesis in an autocrine and also a paracrine manner. Another possibility is that IL-6 induces several growth factors such as hepatocyte growth factor (13) and endothelial growth factor (14), which in turn contribute to restoration of the liver architecture in response to stress-exacerbated liver damage (15). In fact, IL-6 deficiency is known to retard liver regeneration after partial hepatectomy (16).

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