

# Intercellular Adhesion Molecule-1 and CD18 Are Involved in Neutrophil Adhesion and Its Cytotoxicity to Cultured Sinusoidal Endothelial Cells in Rats

SHINICHI SAKAMOTO,<sup>1</sup> TAKESHI OKANOUE,<sup>1</sup> YOSHITO ITOH,<sup>1</sup> KYOKO SAKAMOTO,<sup>2</sup> KENICHI NISHIOJI,<sup>1</sup> YOSHIO NAKAGAWA,<sup>1</sup> NORIMASA YOSHIDA,<sup>2</sup> TOSHIKAZU YOSHIKAWA,<sup>2</sup> AND KEI KASHIMA<sup>1</sup>

The expression of several adhesion molecules is increased on the hepatic sinusoidal endothelial cells (SECs) in various liver diseases. The objective of this study is to assess the roles of intercellular adhesion molecule 1 (ICAM-1) and of CD18 in the interaction between the neutrophils (polymorphonuclear leukocytes [PMNs]) and SECs and in the injury to SECs mediated by PMNs. Rat PMNs was perfused on SECs stimulated with tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) using an *in vitro* flow system. The number of adhered PMNs to SECs and that of PMNs migrated under SECs was counted and the effects of anti-ICAM-1, anti-CD18, and dexamethasone were studied. We also define the effect of these antibodies on the SEC injury mediated by PMNs stimulated with phorbol 12-myristate 13-acetate (PMA) or *N*-formyl-methionyl-leucyl-phenylalanine (fMLP). TNF- $\alpha$  significantly increased the adhesion of PMNs to SECs ( $322 \pm 26$  cells/mm<sup>2</sup>) compared with controls ( $194 \pm 22$  cells/mm<sup>2</sup>). Anti-ICAM-1 and anti-CD18 significantly inhibited the adhesion of PMNs ( $131 \pm 10$  and  $51 \pm 30$  cells/mm<sup>2</sup>, respectively). These antibodies also decreased the migration rate of PMNs (6.0% and 7.9%, respectively) compared with controls (migration rate, 21.2%). The SEC injury induced by PMA- and fMLP-activated PMNs was prevented by anti-ICAM-1 and anti-CD18. The adhesion of PMNs induced by TNF- $\alpha$  was inhibited by the treatment with dexamethasone ( $160 \pm 20$  cells/mm<sup>2</sup>) via a down-regulation of ICAM-1 expression on SECs. The interactions between ICAM-1 and CD18 appeared to be important in the adhesion and the migration of PMNs to SECs. The injury to SECs was induced by the close interaction between the activated PMNs and SECs mediated via ICAM-1 and CD18. (HEPATOLOGY 1997;26:658-663.)

Abbreviations: ICAM-1, intercellular adhesion molecule 1; PMN, polymorphonuclear leukocyte; SEC, sinusoidal endothelial cell; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; HBSS, Hank's balanced salt solution; ELISA, enzyme-linked immunosorbent assay; PMA, phorbol 12-myristate 13-acetate; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine.

From the <sup>1</sup>Third Department of Internal Medicine; and the <sup>2</sup>First Department of Internal Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan.

Received March 12, 1997; accepted April 10, 1997.

Supported in part by grant-in-aid 07457139 from the Ministry of Education, Science and Culture of Japan.

Presented in part at the 1995 Annual Meeting of the American Association for the Study of Liver Disease in Chicago, IL.

Address reprint requests to: Takeshi Okanou, M.D., Third Department of Internal Medicine, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyoku, Kyoto 602, Japan. Fax: 81-75-251-0710.

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Leukocytes must interact with endothelial cells before they can infiltrate the tissue. Specific interactions between the adhesion molecules on the surface membranes of leukocytes and endothelial cells mediate the recruitment of leukocytes into inflammatory sites.<sup>1</sup> Intercellular adhesion molecule-1 (ICAM-1), a member of the immunoglobulin family, is reported to be expressed on endothelial cells and to bind to leukocyte integrins such as Mac-1 (CD11b/CD18) on the neutrophils (polymorphonuclear leukocytes [PMNs])<sup>2</sup> or lymphocyte function-associated antigen 1 (CD11a/CD18) on the lymphocytes.<sup>3</sup> ICAM-1 is present at the basal and luminal sides of endothelial cells and plays a key role in the strong adhesion, as well as the transendothelial migration of human T lymphocytes and PMNs.<sup>4,5</sup> For these local interactions between leukocytes and endothelium, the localized calcium flux is induced in endothelial cells surrounding the intruding leukocyte.<sup>6</sup> Integrin-associated protein (CD47) is involved in calcium regulation in endothelium to be essential for neutrophil migration through endothelium.<sup>7</sup>

Immunohistochemical study has shown these adhesion molecules to be involved in the pathogenesis of various liver diseases. Volpes et al.<sup>8</sup> observed that the expression of ICAM-1 was increased on the sinusoidal endothelial cells (SECs) in inflamed lesions that lymphocyte function-associated antigen 1-positive lymphocytes infiltrated. Burra et al.<sup>9</sup> stated that the increased expression of ICAM-1 and of vascular endothelial adhesion molecule 1 on the hepatic SECs of patients with alcoholic liver cirrhosis might cause hepatic injury. Thus far, there are few studies on the interaction between PMNs and SECs *in vitro*.

Large numbers of PMNs accumulate in the liver tissue of patients with alcoholic hepatitis and septic liver injury associated with the development of liver injury.<sup>10,11</sup> Komatsu et al.<sup>12</sup> showed that the depletion of PMNs by use of anti-neutrophil antibody prevents liver dysfunction in rats with sepsis. These reports suggest that PMNs are important in the pathogenesis of such disorders. The present study addresses the hypothesis that an ICAM-1-CD18-mediated interaction may be involved in the adhesion of PMNs to SECs in PMN-mediated liver injury. We have used an *in vitro* flow system that allows for the dynamic analysis of the spatial and temporal details of cell-to-cell interactions<sup>13,14</sup> and have assessed the interaction between PMNs and SECs stimulated with tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ).

Endothelial cell injury elicited by activated leukocytes is known to be the initial stage of various inflammatory diseases. The adhesion of leukocytes to the endothelium has

been recognized as an important process in leukocytes causing endothelial cell injury.<sup>15</sup> This injury is mainly caused by the active oxygen species or several proteases released by activated leukocytes.<sup>16,17</sup> Then, we investigated the role of adhesion molecules, radical scavengers, and a protease inhibitor on PMN-mediated injury to SECs. Additionally, we evaluated the interaction between PMNs and SECs and the expression of ICAM-1 on SECs in the presence of dexamethasone because glucocorticoids are administered to patients with alcoholic hepatitis.

#### MATERIALS AND METHODS

**Animals.** Male Wistar rats (Shimizu Animal, Inc., Kyoto, Japan) (weight range, 200-250 g) were used in this study. They were fed a standard laboratory diet and water ad libitum under standard laboratory conditions. Procedures were performed according to the guidelines of the "Guide for the Care and Use of Laboratory Animals" from the National Institutes of Health.

**Isolation and Culture of Rat SECs.** SECs were obtained from male Wistar rats according to a previously described method<sup>18</sup> with a slight modification. Briefly, the liver was perfused via the portal vein with Hank's solution that was free of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  and contained 0.01% ethylenediaminetetraacetic acid and 10 U/mL of heparin. The liver was perfused subsequently with Hank's balanced salt solution (HBSS) supplemented with 0.05% collagenase. The liver was then removed, cut into pieces, and passed through a mesh to remove cell debris. Low-speed centrifugation was performed twice to separate the hepatocytes. The suspension of nonparenchymal cells was then introduced into a centrifugal elutriation rotor (Hitachi Koki Co., Ltd., Ibaragi, Japan) to isolate the SECs. SECs were suspended in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal calf serum (GIBCO Laboratories, Grand Island, NY),  $10^{-7}$  mol/L dexamethasone (Sigma Chemical Co., St. Louis, MO), and 0.1% gentamycin sulfate (Sigma Chemical Co.) at a density of  $2 \times 10^6$  cells/mL. An aliquot (1.0 mL) of the endothelial cell suspension was cultured on a  $24 \times 50$ -mm glass coverslip (Matsunami Glass Industries, Ltd., Osaka, Japan) and coated with type I C collagen (Nitta Gelatin, Inc., Osaka, Japan) for 2 hours at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . Coverslips were then rinsed and cultured in a medium free of dexamethasone for another 10 hours for the adhesion assay. To measure the expression of ICAM-1 by enzyme-linked immunosorbent assay (ELISA), 100  $\mu\text{L}$  of the endothelial cell suspension ( $2 \times 10^5$  cells/well) was cultured on a 96-well plate coated with type I C collagen (Corning 25860 CPL 1; Iwaki Glass, Tokyo, Japan) as described previously. SECs were identified morphologically by scanning electron microscopy and histochemically by use of DiI-acetylated low-density lipoprotein labeled with the fluorescent probe.<sup>19</sup> About 92% of cultured endothelial cell fraction showed the characteristic features of pores and sieve plates and took up acetylated low-density lipoprotein. Immunohistochemical staining for anti-rat monocyte/macrophage antibody (ED1; Chemicon International Inc., Temacula, CA) showed that 4% to 6% of cultured endothelial cell fraction contained liver macrophages.

**Isolation of Rat PMNs.** Rat PMNs were isolated from the peritoneal fluid 4 hours after the intraperitoneal injection of 15 mL of 0.1% glycogen obtained from oysters (Sigma Chemical Co.). After washing with HBSS, the cells were suspended in HBSS at a density of  $1 \times 10^6$  cells/mL and maintained at  $4^\circ\text{C}$  for 1 hour. Giemsa staining showed >95% of the isolated cells to be PMNs. Flow-cytometrical analysis showed that 96% of the cell fraction isolated from the peritoneal fluid expressed CD18 (WT.3; Seikagaku Corp., Tokyo, Japan) on their own surface (data not shown) as previously reported.<sup>20</sup>

**Adhesion Assay.** The adhesion and migration of PMNs on SECs were assessed using an *in vitro* flow system.<sup>13,14</sup> The chamber consisted of a collagen-coated coverslip with a confluent monolayer of SECs that was attached to a polycarbonate base. The space between

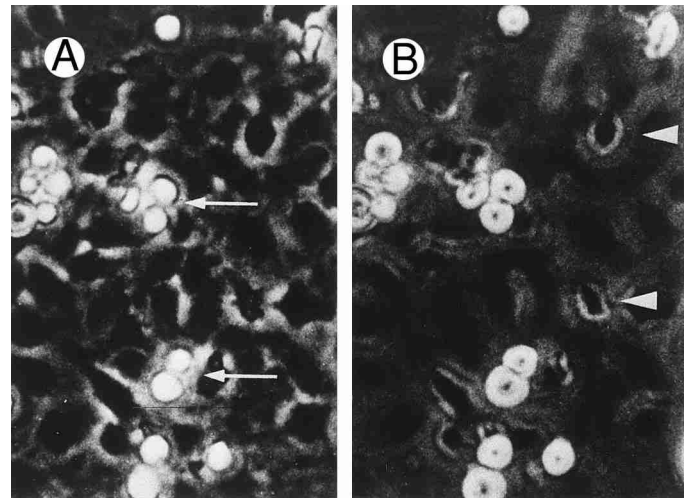


FIG. 1. Phase-contrast micrographs of adhesion and migration of neutrophils to monolayer of hepatic SECs. A is focused on the luminal surface of the monolayer. B is focused just beneath the monolayer. The neutrophils adherent on the monolayer (arrow) were rounded up and appeared as bright objects, and those migrated to the subluminal space (arrowhead) became spread and phase-dark. (Original magnification  $\times 200$ .)

the coverslip and the base was held approximately  $250 \mu\text{m}$  apart by a silastic rubber gasket. The flow rate was controlled with a syringe pump (Iwaki Glass Co., Ltd.). Suspensions of PMNs were passed through the chamber at a wall shear stress of  $0.5 \text{ dyne/cm}^2$  and  $37^\circ\text{C}$ . Shear stress,  $\tau$  in  $\text{dyne/cm}^2$ , was calculated from the following equation:

$$\tau = 3\mu Q/2ba^2,$$

where  $\tau$  is the wall shear stress,  $\mu$  is the viscosity of water at  $37^\circ\text{C}$ , which is used as an approximation of the viscosity of HBSS (0.007 poise),  $Q$  is volumetric flow rate,  $a$  is the half channel height, and  $b$  is the channel width. The wall shear stress in the hepatic sinusoid was calculated from leukocyte velocity and the diameter of the microvessels that were measured by intravital fluorescence microscopy used by Vollmar et al.<sup>21</sup> The wall shear stress in the hepatic sinusoid was lower than that in venule because of its slow blood flow. A wall shear stress of  $0.5 \text{ dyne/cm}^2$  is supposed to be within pathophysiological condition in hepatic sinusoids, based on their findings. SECs on the coverslips were stimulated with 100 U/mL of recombinant human TNF- $\alpha$  (rhTNF- $\alpha$ ) (Dainippon Pharmaceutical Co., Osaka, Japan) for 4 hours, mounted in flow chambers, and perfused for 1 minute with HBSS (rinse step) followed by an 8-minute perfusion with a suspension of PMNs (perfusion step). In some experiments, an antibody against human TNF- $\alpha$  (Dainippon Pharmaceutical Co.) was added. The interaction between PMNs and SECs was observed by phase-contrast microscopy (Olympus Co., Ltd., Tokyo, Japan) and was recorded on a videotape. The number of PMNs that remained in contact with the endothelial cell monolayer was determined in six digitized frames of different fields of view from the last minute (7-8 minute) of the perfusion step and was expressed as the number of cells that adhered or migrated per square millimeter. The PMNs that adhered to the monolayer possessed spherical geometry and appeared as phase-bright objects, whereas those that migrated beneath the monolayer were flat and appeared as phase-dark objects, as described previously<sup>14</sup> (Fig. 1). The migration rate was quantitated as follows:

Migration rate (%)

$$= \text{Migrated PMNs} \times 100 \div (\text{Adhered PMNs} + \text{Migrated PMNs}).$$

In some experiments, pretreatment of SECs with anti-rat ICAM-

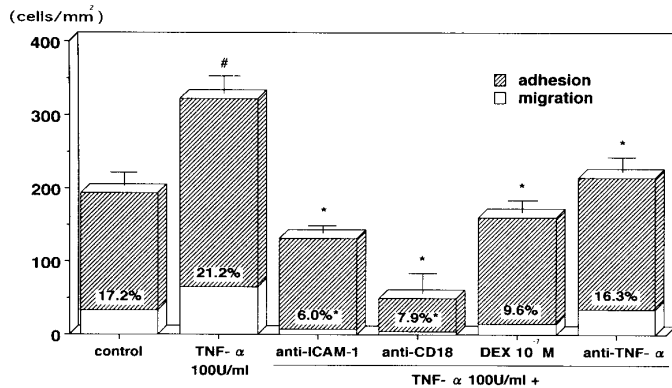


FIG. 2. Effects of monoclonal antibodies to ICAM-1 or CD18 and dexamethasone (DEX;  $1 \times 10^{-7}$  mol/L) and anti-TNF- $\alpha$  on neutrophil adhesion and migration to SECs induced by rhTNF- $\alpha$  (100 U/mL for 4 hours). Monoclonal antibodies were added at a final concentration of 5  $\mu$ g/mL. Each value represents means  $\pm$  SEM of three experiments performed in duplicate. The percent shows the migration rate of neutrophils determined by the following equation:

Migration Rate (%)

$$= \text{Migrated PMNs} \times 100 \div (\text{Adhered PMNs} + \text{Migrated PMNs}).$$

\* $P < .05$  compared with control. \* $P < .05$  compared with 100 U/mL TNF- $\alpha$  group without monoclonal antibody or dexamethasone.

1 monoclonal antibody (1A29; 5  $\mu$ g/mL, Seikagaku Corp.) or pretreatment of isolated PMNs with anti-rat CD18 (5  $\mu$ g/mL) for 20 minutes at 37°C was performed.

**Expression of ICAM-1 on SECs.** The expression of ICAM-1 on SECs was measured by cellular ELISA. SECs cultured on a 96-well plate coated with type I C collagen were incubated with 1, 10, 100, and 1,000 U/mL of rhTNF- $\alpha$  for 4 hours and fixed in 4% paraformaldehyde. In some experiments, an antibody against human TNF- $\alpha$  was added. To investigate the effect of dexamethasone on ICAM-1 expression, serial concentrations of dexamethasone were added in some experiments. Three washes were done in phosphate-buffered saline containing 0.1% bovine serum albumin. Nonspecific reactions were blocked by incubation for 30 minutes with 1% normal goat serum (Sigma Chemical Co.). After an additional wash, cells were incubated for 1 hour with 50  $\mu$ L of anti-rat ICAM-1 monoclonal antibody at a dilution of 1:1000. After three washes with phosphate-buffered saline that contained bovine serum albumin, the cells were incubated for 1 hour with a peroxidase-labeled antibody against mouse immunoglobulin G (Seikagaku Corp.). Peroxidase staining was then performed with *o*-phenylenediamine (Sigma Chemical Co.) and H<sub>2</sub>O<sub>2</sub>. Absorbance at 490 nm was measured by an Immunoreader (Multiskan; Labosystems, Helsinki, Finland). The microplates were subsequently washed in tap water before 50  $\mu$ L of 0.5% crystal violet was added for 5 minutes. After a thorough wash in tap water, 100  $\mu$ L of 33% acetic acid was used to solubilize the nuclear stain, and absorbance at 570 nm was measured, representing the actual cell count per well. The expression of ICAM-1 was provided as the ratio between specific protein measurement and cell density.

**Assay of Injury to the SECs.** <sup>51</sup>Cr Release was used as an index of the injury of SECs. This method has been used for the assay of cell- or chemical-mediated cytotoxicity.<sup>22,23</sup> In brief, SECs were seeded into a 48-well culture plate coated with type I C collagen and were incubated with culture medium containing 15  $\mu$ Ci Na<sup>51</sup>CrO<sub>4</sub>/mL for 12 hours. SECs were washed twice with culture medium to remove unincorporated radioactivity. After stimulating the SECs with rhTNF- $\alpha$  (100 U/mL for 4 hours),  $1 \times 10^5$  cells of rat PMNs in a culture medium containing 10 ng/mL of phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co.) or 100 nmol/L of *N*-formyl-methionyl-leucyl-phenylalanine (fMPL) (Sigma Chemical Co.) was

added to each well and incubated for 6 hours in the presence or absence of the following: anti-ICAM-1 antibody, anti-CD18 antibody, catalase (1,000 U/mL) (Sigma Chemical Co.), superoxide dismutase (1,000 U/mL) (Seikagaku Corp.), and aprotinin (1,000 U/mL) (Sigma Chemical Co.). In some experiments,  $2 \times 10^4$  cells or  $5 \times 10^4$  cells of PMNs were added to the well ( $\frac{1}{5}$  PMN group and  $\frac{1}{2}$  PMN group, respectively). <sup>51</sup>Cr Radioactivity released from the injured SECs was measured. The percentage of specific <sup>51</sup>Cr release, an index of injury to SECs, was quantitated as follows:

% Specific <sup>51</sup>Cr Release

$$= (\text{Experimental Count} - \text{Spontaneous Count}) \times 100 \div (\text{Total Count} - \text{Spontaneous Count}).$$

The total count was taken as that for the well receiving 0.2% Triton X-100 containing 0.1N NaOH and spontaneous count for that with culture medium only. The experimental count represents the count for <sup>51</sup>Cr release induced by PMA- or fMPL-stimulated PMNs with or without reagents.

**Statistical Analysis.** Values are expressed as means  $\pm$  SEM. Data were analyzed by analysis of variance and Student's *t* test, with Bonferroni's correction for multiple comparisons. An unpaired two-tailed Student's *t* test was used to compare the migration rates and percent inhibitions. A *P* value of  $< .05$  was considered statistically significant.

## RESULTS

**Adhesion and Migration of PMNs to TNF- $\alpha$ -Stimulated SECs.** The number of adherent PMNs to the monolayers of SECs was increased significantly by stimulation with 100 U/mL of rhTNF- $\alpha$  (Fig. 2). This was inhibited by the addition of neutralizing anti-TNF- $\alpha$  antibody. To investigate the effects of ICAM-1 and CD18 on the adhesion of PMNs, we assessed the adhesion of PMNs after pretreating the SECs with anti-rat ICAM-1 antibody or after pretreating the PMNs with anti-CD18 antibody. Five micrograms per milliliter each of the anti-ICAM-1 and anti-CD18 antibody significantly reduced the number of adherent PMNs compared with that observed in the absence of an antibody (Fig. 2). Each antibody significantly reduced the migration rate of PMNs ( $P < .05$ ).

**Effect of TNF- $\alpha$  on ICAM-1 Expression.** To determine whether the increase in PMN adherence induced by rhTNF- $\alpha$  was related to an increased expression of ICAM-1, we determined the expression of ICAM-1 on SECs by cellular ELISA. TNF- $\alpha$  enhanced the expression of ICAM-1 on SECs in a dose-dependent manner. The presence of anti-TNF- $\alpha$  antibody inhibited this effect (Fig. 3).

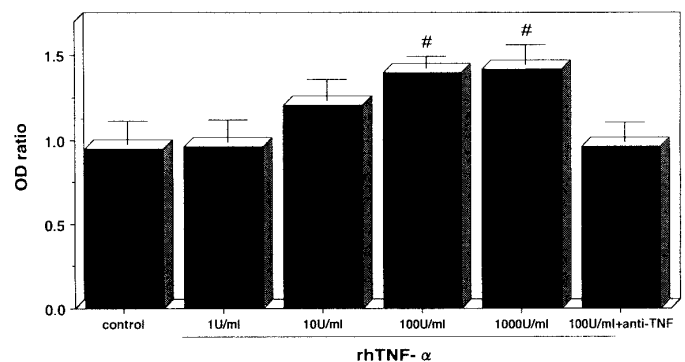


FIG. 3. Effects of rhTNF- $\alpha$  on the expression of ICAM-1 on SECs. ICAM-1 expression on SECs stimulated with rhTNF- $\alpha$  for 4 hours was measured by ELISA. Each value represents means  $\pm$  SEM of three experiments performed in triplicate. \* $P < .05$  compared with control.

**PMN-Mediated Injury to SECs.** The injury to SECs caused by PMA- or fMLP-stimulated PMNs are shown in Fig. 4. Culture with the PMA- or fMLP-stimulated PMNs induced the release of  $^{51}\text{Cr}$  from SECs. The reduction in the number of cocultured PMNs resulted in the decrease of this endothelial cytotoxicity. Anti-ICAM-1 and anti-CD18 antibody each suppressed both PMA- and fMLP-induced injury to SECs. The addition of catalase, which breaks down hydrogen peroxide, suppressed PMA-induced injury to SECs but not fMLP-induced injury to SECs. The addition of superoxide dismutase, which breaks down superoxide anion, had little effect on both SEC injuries. The cytotoxicity by fMLP-stimulated PMNs was significantly abrogated by the addition of 1,000 U/mL of aprotinin, a protease inhibitor.

**Effects of Dexamethasone on the Expression of ICAM-1 and on PMN and SEC Interactions.** To investigate the anti-inflammatory mechanism of dexamethasone, we assessed its effect on the expression of ICAM-1 expression on SECs. Dexamethasone suppressed the overexpression of ICAM-1 induced by rhTNF- $\alpha$  dose-dependently (Fig. 5). Concentrations of dexamethasone of  $\geq 1 \times 10^{-8}$  mol/L showed a significant effect. As shown in Fig. 1,  $1 \times 10^{-7}$  mol/L of dexamethasone significantly inhibited the adhesion of PMNs to SECs. Although the

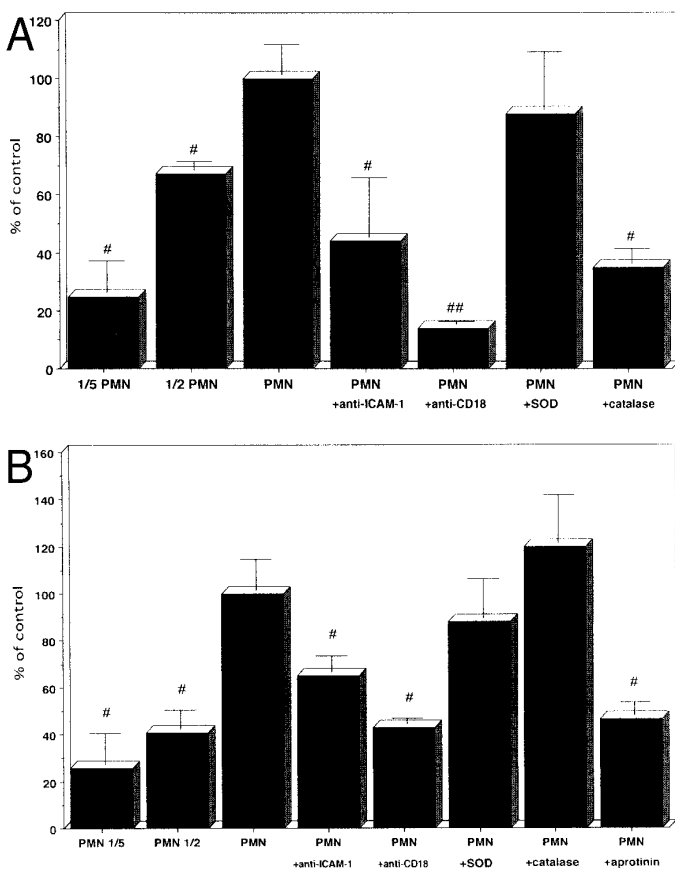


FIG. 4. Role of adhesion and neutrophil-derived oxidants in SEC injury induced by PMNs.  $^{51}\text{Cr}$  Release was assessed at 6 hours after incubation. Each value represents means  $\pm$  SEM of three experiments performed in triplicate and is expressed as the percentage of the control. The control represents cytotoxicity induced by  $1 \times 10^5$  cells of rat PMNs. The  $\frac{1}{5}$  PMN and the  $\frac{1}{2}$  PMN represent cytotoxicity induced by  $2 \times 10^4$  cells and  $5 \times 10^4$  cells of PMNs, respectively. # $P < .05$  compared with PMN group (control) and ## $P < .01$  compared with PMN group.

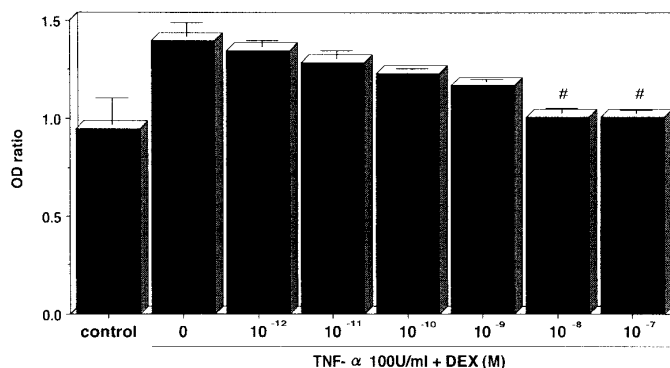


FIG. 5. Effects of dexamethasone (DEX) on ICAM-1 expression on SECs. ICAM-1 expression was assessed by ELISA on TNF- $\alpha$ -stimulated sinusoidal endothelial cells in the presence of serial concentrations of dexamethasone. Each value represents means  $\pm$  SEM of three experiments performed in triplicate. # $P < .05$  compared with 100 U/mL TNF- $\alpha$  group without dexamethasone.

migration rate of PMNs tended to decrease in the presence of  $1 \times 10^{-7}$  mol/L of dexamethasone, the effect was not significant.

## DISCUSSION

Although numerous PMNs infiltrate the liver including the hepatic sinusoids in alcoholic- or sepsis-related liver injury, the mechanism is not well understood. To clarify the mechanism of the liver injury mediated by PMNs, we focused on the interaction between ICAM-1 and CD18. This study showed that the interaction between ICAM-1 and CD18 was involved in PMN adhesion to and migration through SECs and that this interaction was important for the injury to SECs induced by PMA- and fMLP-activated PMNs.

The role of the adhesion molecules in the interaction between the leukocytes and endothelial cells was pathophysiologically investigated *in vivo* by using an intravital microscope in the mesenteric circulation of rats<sup>24</sup> and *in vitro* by using human umbilical vein endothelial cells in an *in vitro* adhesion assay.<sup>13,14</sup> These experiments showed that ICAM-1 is important for the adhesion of PMNs to the endothelium. CD11b/CD18 monoclonal antibody is reported to prevent the hepatic infiltration of PMNs and the necrosis of hepatocytes induced by the intraperitoneal injection of endotoxin.<sup>25</sup> This study suggests that an ICAM-1-CD11b/CD18-mediated interaction is important for the accumulation of PMNs in the liver to induce hepatocellular necrosis.

We investigated the roles of ICAM-1 and CD18 in the adhesion of PMNs to SECs and the migration of PMNs through SECs by means of an *in vitro* flow system. This system provides for the dynamic analysis of the interactions between the PMNs and SECs, such as rolling, adhesion, and migration. We showed that the pretreatment of SECs with rhTNF- $\alpha$  induced the adhesion of PMNs and that the anti-ICAM-1 and anti-CD18 antibody each inhibited the increase in PMN adhesion (Fig. 2). Each antibody also prevented the migration of PMNs beneath the SEC monolayer. These results are consistent with those of Issekutz et al.,<sup>26</sup> who showed that the transendothelial migration of PMNs beneath a human umbilical vein endothelial cell monolayer was blocked by >90% by anti-CD18 and partially blocked by anti-ICAM-1. Our results indicate that ICAM-1 and CD18

both play a key role in the adhesion and the migration of PMNs in the hepatic sinusoids.

It is reported that ICAM-1 is weakly expressed on SECs of normal rat *in vivo*.<sup>27</sup> Our cellular ELISA showed that ICAM-1 was expressed on SECs in basal conditions *in vitro* and that rhTNF- $\alpha$  increased its expression in a dose-dependent manner. Ohira et al. reported that serum levels of TNF- $\alpha$  and ICAM-1 expression on SECs were increased in rats treated with lipopolysaccharide.<sup>28</sup> Our *in vitro* study supports their observation that TNF- $\alpha$  might induce PMN infiltration in hepatic sinusoids by the induction of ICAM-1 on SECs.

The injury to the endothelial cells elicited by activated leukocytes is the initial event in various forms of inflammation. It is mainly caused by the active oxygen species or several proteases released by activated PMNs.<sup>16,17,29</sup> Komatsu et al. reported that the serum from rat treated with galactosamine-lipopolysaccharide enhanced the release of oxygen-derived free radicals by PMNs that contribute to the hepatic injury.<sup>12</sup> We showed that both PMA- and fMLP-activated PMNs induced SEC injury in a PMN/SEC ratio-dependent manner. This shows that PMNs infiltrating hepatic sinusoids could induce the endothelial cell damage that causes the disturbance of sinusoidal microcirculation.

Weiss has stated that oxygen radicals and proteases cooperate or interact in the tissue damage caused by PMNs.<sup>16</sup> To evaluate the role of the active oxygen species or proteases in PMA- or fMLP-induced SEC injury, assays were performed in the presence of reactive oxygen scavengers or a protease inhibitor. In the present study, we showed that catalase prevented PMA-induced SEC injury, which seemed to be induced by hydrogen peroxide but not fMLP-induced SEC injury. This finding is supported by Fujita et al.<sup>30</sup> who showed that PMA-stimulated PMNs injure human umbilical vein endothelial cells by producing hydrogen peroxide and by inducing the increase in the level of intercellular peroxides in endothelial cells. In addition, we showed that fMLP-induced PMN-mediated SEC cytotoxicity was inhibited by aprotinin but not by reactive oxygen scavengers. Aprotinin is a broad-spectrum protease inhibitor that has been used in the management of experimental endotoxin and septic shock.<sup>31</sup> This showed that fMLP-stimulated PMNs cause SEC injury mainly by release of PMN protease. Endotoxin-related liver injury involves SEC injury caused by infiltrated PMNs<sup>28</sup> and is inhibited by protease inhibitors.<sup>32</sup> Our *in vitro* study showed that SEC injury caused by activated PMNs involved active oxygen species or proteases.

The present study also showed that anti-ICAM-1 and anti-CD18 each protected against SEC injury induced by both PMA- and fMLP-activated PMNs, showing that these adhesion molecules were crucial for SEC injury mediated by different mechanisms, the active oxygen species and proteases. It is reported that activated neutrophils produce hydrogen peroxide and that the CD18-mediated interaction with endothelial cells increases hydrogen peroxide production by neutrophils.<sup>33</sup> The treatment with anti-ICAM-1 or CD18 antibody not only inhibited the close contact between SECs and neutrophils but also might decrease the production of active oxygen species. Injury to SECs leads to vascular protein leakage and to an increase in the extravasation of PMNs. Migrated PMNs achieve access to the hepatocytes. Inflammatory cytokines promote the expression of ICAM-1 on the hepatocytes and increase the adhesion of the lymphocytes to the hepatocytes.<sup>34</sup> Essani et al.<sup>35</sup> reported that anti-ICAM-1 reduced

extravasation of PMNs to hepatocytes and inhibited liver injury. This is consistent with our finding that anti-ICAM-1 reduced the migration of PMNs beneath the SEC monolayer. The protective effect of anti-ICAM-1 against the hepatic injury induced by PMNs may be explained by our results showing that anti-ICAM-1 inhibited the close contact between the PMNs and SECs and reduced the injury to SECs induced by activated PMNs.

The involvement of leukocyte adhesion in the pathogenesis of hepatic injury related to alcoholism or sepsis has clinical relevance. It is reported that corticosteroids improve the survival in patients with severe alcoholic hepatitis<sup>36</sup>; however, its mechanism has not been clarified yet. We investigated the effects of dexamethasone on TNF- $\alpha$ -induced overexpression of ICAM-1 on SECs and on the interaction between PMNs and SECs to clarify the mechanism of the anti-inflammatory effects of dexamethasone in PMN-mediated liver injury. Dexamethasone inhibited the expression of ICAM-1 on SECs in a dose-dependent manner. The effects of dexamethasone at concentrations of  $>1 \times 10^{-8}$  mol/L were statistically significant. In an *in vitro* adhesion assay, at a concentration of  $1 \times 10^{-7}$  mol/L, dexamethasone significantly inhibited the adhesion to SECs and tended to reduce the migration rate of PMNs. During rejection of liver allografts, corticosteroids reduce the expression of ICAM-1 on hepatic endothelium and hepatocytes and prevent the rejection.<sup>37</sup> Our findings support the hypothesis that corticosteroids prevent the hepatic accumulation of PMNs, in part, by down-regulating ICAM-1 on SECs.

In summary, the interaction between ICAM-1 and CD18 was involved in the adhesion and migration of PMNs in hepatic sinusoids mediated by TNF- $\alpha$ . This interaction was the key to the SEC injury induced by activated PMNs. Dexamethasone inhibited the adhesion of PMNs to SECs by reducing the expression of ICAM-1 on SECs. Blocking the interaction of ICAM-1-CD18 may point to a therapeutic strategy for preventing liver injury mediated by PMNs.

**Acknowledgment:** The authors thank Nanako Kaibara for her excellent technical assistance in this study.

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