Role of CD18-ICAM-1 in the entrapment of stimulated leukocytes in alveolar capillaries of perfused rat lungs

TAKUYA AOKI,1 YUKIO SUZUKI,3 KAZUMI NISHIO,1 KOUICHI SUZUKI,1 ATSUSHI MIYATA,1 YUTAKA IIGOU,2 HIROSHI SERIZAWA,3 HARUKUNI TSUMURA,4 YUZURU ISHIMURA,2 MAKOTO SUEMATSU,2 AND KAZUHIRO YAMAGUCHI1

Departments of 1Internal Medicine and 2Biochemistry, School of Medicine, Keio University, Tokyo 160; 3Department of Internal Medicine, Kitasato Institute Hospital, Tokyo 108; 4Biomedical Department, Sankei Corporation, Tokyo 113; and 5New Product Research Laboratories IV, Daiichi Pharmaceutical Company, Tokyo 134, Japan.

Aoki, Takuya, Yukio Suzuki, Kazumi Nishio, Kouichi Suzuki, Atsushi Miyata, Yutaka Iigou, Hiroshi Serizawa, Harukuni Tsumura, Yuzuru Ishimura, Makoto Suematsu, and Kazuhiro Yamaguchi. Role of CD18-ICAM-1 in the entrapment of stimulated leukocytes in alveolar capillaries of perfused rat lungs. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H2361–H2371, 1997.—This study aimed to examine the behavior of stimulated leukocytes in the pulmonary microcirculation. The leukocyte-endothelium interaction was visualized under physiological shear rates in perfused rat lungs using high-speed confocal laser video microscopy. Leukocytes labeled with carboxyfluorescein were stimulated with cytokine-induced neutrophil chemoattractant (CINC/gro), which caused leukocyte rolling and adhesion to venules, whereas both were sequestered in capillaries. Approximately 50% of stimulated leukocytes showed a transient cessation of movement in pulmonary capillaries. The CINC/gro stimulation, which inhibited leukocyte rolling and adhesion to mesenteric venules, reduced leukocyte velocity and increased leukocytes in pulmonary capillaries. Pretreatment with monoclonal antibodies against intercellular adhesion molecule-1 (ICAM-1) or CD18 attenuated these changes. Confocal microscopy revealed constitutively expressed ICAM-1 not only in venules but also abundantly in capillary networks. These results suggest that selectin-independent, CD18-ICAM-1-dependent capillary sequestration is one of the major mechanisms by which activated leukocytes accumulate in the lungs.

METHODS

Reagents used. Rat cytokine-induced neutrophil chemoattractant (CINC/gro) is a peptide possessing biological activity analogous to those of the human interleukin-8 (IL-8) family (32) and was a generous gift from K. Watanabe (Institute of Cytosignal Research, Tokyo, Japan). MAbs against CD18 (WT-3), against ICAM-1 (1A29), and against leukocyte adhesion molecules analogous to those of the human interleukin-8 (IL-8) family (32) and was a generous gift from Dr. K. Watanabe (Institute of Cytosignal Research, Tokyo, Japan). MAbs against CD18 (WT-3), against ICAM-1 (1A29), and against L-selectin (HRL-4) were generously provided by M. Miyasaka (Dept. of Bioregulation, Osaka University Medical School, Biomedical Research Center, Osaka, Japan). Fluorescein isothiocyanate (FITC), FITC-dextran (mol wt 145,000), FITC-labeled anti-mouse immunoglobulin G (IgG) antibody, and mouse IgG were purchased from Sigma (St. Louis, MO), and carboxyfluorescein diacetate succinimidyl ester (CFSE) was obtained from Molecular Probes (Eugene, OR). Anti-CD18 monoclonal antibody (MAb) 60.3 inhibited neutrophil emigration and edema formation induced by Streptococcus pneumoniae in vivo and in vitro (20).

In pulmonary microvessels, however, whether these adhesion molecules are required for leukocyte accumulation is still controversial (4, 10). Worthen et al. (35) reported that stimulated neutrophils remain in pulmonary capillaries as a result of decreased cellular deformability. Vedder et al. (28) demonstrated that blockade of the CD11/CD18 glycoprotein adherence complex by the monoclonal antibody (MAb) 60.3 inhibited neutrophil accumulation in the gut but did not inhibit the pulmonary sequestration of neutrophils in ischemia-reperfusion injury. In addition, recent data obtained from studies utilizing P-selectin and ICAM-1 double mutant mice showed that peritoneal neutrophil emigration and edema formation induced by Streptococcus pneumoniae was attenuated in mutant mice, whereas pulmonary neutrophil emigration and edema formation occurred as observed in the wild-type mice (4). These results suggest little, if any, involvement of these adhesion molecules in the mechanism underlying pulmonary leukocyte accumulation and in the subsequent lung injury. On the other hand, several previous studies have shown that anti-CD18 MAb attenuated lung injuries induced by tumor necrosis factor (17), gram-negative sepsis (31), and zymosan-activated plasma (8).

Although the discrepancy concerning the contribution of the CD18-ICAM-1 interaction to neutrophil accumulation in the lung appears to reflect the varied experimental protocols and choices of animal species used, the crucial factors leading to this controversy are possible differences in wall shear rates and circulating leukocyte numbers among the experimental models as well as unique features of the interaction between activated leukocytes and endothelium in the pulmonary microcirculation. We hypothesized that sequential multistep leukocyte-endothelium interactions are not applicable to lung microvessels. Therefore, the aim of the present study was to examine the behavior of circulating leukocytes and their adhesion mechanisms in the rat pulmonary microcirculation perfused ex vivo under controlled flow conditions.

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The role of CD18-dependent intercellular adhesion molecule-1 (ICAM-1) interaction in the entrapment of stimulated leukocytes in alveolar capillaries of perfused rat lungs.
was from Molecular Probes (Eugene, OR). MAb (2H5s) against sialyl Lewis X-like carbohydrates (SLex) was provided by Dr. T. Tamatani (Pharmaceutical Basic Research Laboratories, J anpan Tobacco, Yokohama, J anpan) (27).

Animal preparation. Specific pathogen-free male Sprague-Dawley rats (Sankyo Laboratory Service, Tokyo, J anpan), 8 wk of age and weighing 250–300 g, were used. All of the following experimental protocols were approved by the animal committee of Keio University School of Medicine, Tokyo, J anpan. Animals were anesthetized with pentobarbital sodium (50 mg/kg ip). The trachea was cannulated and connected to a ventilator, then ventilated at a tidal volume of 10 ml/kg and a respiratory rate of 60 breaths/min. Lungs were exposed by median sternotomy, and blood was withdrawn from the heart. The trachea was ligated at the level of one tidal volume above functional residual capacity and fixed on a microscope stage in the supine position. The main pulmonary artery and left atrium were catheterized. Pulmonary arterial pressure was measured with a pressure transducer (SEN-6102M; Nihon Kohden, Tokyo, Japan) and monitored continuously during the experiment. The pH was maintained between 7.38 and 7.42.

The perfusate gas tension and pH were measured using a 1306 blood-gas analyzer (Instrumentation Laboratory) at the beginning of and at intervals during each experiment. The pH was maintained between 7.38 and 7.42. The lung was humidified, and the surface temperature was maintained at 37 ± 0.5°C. The left lobar lung of the lung was observed under an in vivo microscopic system (SO-MI, Sankei, Tokyo, J anpan), employing normal and fluorescence objective lenses (×10, ×20, ×40), connected to a closed-circuit video system. The in vivo microscopic system has three lights. The first is a normal lamp light (Techno Light KTS-150, Kenko, Tokyo, J anpan), the second a xenon lamp light (Nikon, Tokyo, J anpan) for fluorescence imaging, and the third a laser power supply (Omnichrome, Chino, CA) for confocal imaging. The image was displayed with a high-sensitivity charge-coupled device camera (TEC-470, Optronics) or a high-sensitivity intensified imager (II) camera (EktaPro intensified imager, Kodak, San Diego, CA) and color video monitor (PVM-1444Q, Sony, Tokyo, J anpan). The image was recorded on videotape with a tape recorder (SVQ-260, Sony). To determine the diameters of arterioles, capillaries, and venules and to analyze the high-speed movement of leukocytes and erythrocytes inside vessels, a confocal laser scanning microscope (Yokokawa, Tokyo, J anpan) was used. Views of high-speed movements of the cells were displayed by using the dynamic confocal laser scanning microscope with the II camera and stored in a high-speed video recorder system (EktaPro TR6,000 system, Kodak). Velocities of leukocytes and erythrocytes were recorded at a rate of 250 frames/s using the high-speed video system. Leukocytes that remained in the same internal portions of capillaries were excluded from the speed analysis.

Visualization of vessel networks, erythrocytes, and leukocytes. To visualize vessel networks, we administered FITC-dextran (mol wt 145,000), at a final concentration of 0.015%, into the perfusion circuit. Erythrocytes were labeled with FITC. Rat blood was diluted with phosphate-buffered saline (PBS) and centrifuged. The erythrocyte pellet was then diluted with PBS. FITC was added at a final concentration of 0.1 mg/ml. After a 30-min incubation at 37°C, the solution was centrifuged and diluted with 5 ml of PBS. Thereafter, 1 ml of the dilute solution was administered into the perfusion circuit, when necessary. Leukocytes were labeled with CFSE according to our previously described method (24). Briefly, the CFSE solution (1.0 mg/ml) diluted with 1.5 ml of physiological saline was injected at 0.3 ml/min into the femoral veins of the donor rats. After a 30-min incubation in vivo, blood samples were collected by heart puncture. To avoid unnecessary activation of the naive cells, which can evoke shedding of L-selectin, no further cell separation was carried out. These procedures allowed us to obtain blood samples for perfusion in which 28.4 ± 3.9, 73.3 ± 7.5, and 3.6 ± 2.5% of leukocytes were neutrophils, lymphocytes, and monocytes, respectively. When used to study the pulmonary microcirculation, the blood samples containing CFSE-labeled leukocytes were directly injected into the perfusion circuit of the recipient rats to give a final leukocyte concentration of 400–500 cells/µl. In separate sets of experiments, the blood samples containing CFSE-labeled leukocytes were used to examine leukocyte behavior in the mesenteric microcirculation, as described below.

Experimental groups. We designed four experimental groups: 1) control, 2) rat IL-8, 3) anti-CD18, and 4) anti-ICAM-1. For the control group (n = 6), CFSE-labeled leukocytes and perfused rat lungs had no pretreatment. For the rat IL-8 group (n = 6), blood including CFSE-labeled leukocytes was incubated with 10 nM CINC/gro at 37°C for 10 min just before administration into the perfusion circuit. For the anti-CD18 group (n = 6), blood including CINC/gro-activated CFSE-labeled leukocytes (same as the rat IL-8 group) was treated with WT-3 for 30 min at room temperature at a final concentration of 50 µg/ml. The blood was then administered into the perfusion circuit. For the anti-ICAM-1 group (n = 4), 1A29 was administered into the perfusion circuit at a perfusate concentration of 10 µg/ml. After a 10-min perfusion period, blood including CINC/gro-activated CFSE-labeled leukocytes (same as the rat IL-8 group) was administered into the perfusion circuit. Separately, we conducted a series of experiments using isotype-matched mouse IgG samples as a control group for those treated with anti-CD18 or anti-ICAM-1 MAbs. CFSE-labeled leukocytes were stimulated with CINC/gro and incubated with mouse IgG at a final concentration of 50 µg/ml for 30 min. We also administered
mouse IgG at a final concentration of 10 μg/ml over 10 min into the perfused rat lung circuit.

Roles of selectins in normal pulmonary leukocyte-endothelial interactions were examined by using a MAb 2H5 (n = 4), which recognizes SLeX and blocks its function to attenuate L-, P-, and E-selectin-mediated cell adhesion (26, 27). Because L- and P-selectins might be involved, under our experimental conditions (L-selectin on leukocytes and P-selectin on pulmonary endothelial cells and the small population of platelets in the perfusate), 2H5 was added to the perfusate at a final concentration of 20 μg/ml, known to be sufficient to abolish selectin-dependent cell adhesion in vitro (27). 2H5 was also added to the leukocyte suspension to block the possible interaction of the neutrophil SLeX with P-selectin at the same MAb concentration. Blood including CFSE-labeled leukocytes was incubated with 2H5 for 30 min at room temperature, and the perfused rat lung was also treated with 2H5 for 10 min.

Histological examination. A catheter was inserted into the main bronchus after each experiment, the duration of which was 30 min after injection of CFSE-labeled leukocytes. The perfused rat lung was then fixed with Formalin and embedded in paraffin. Six sections were cut at the same interval from the bottom of the lung and stained with hematoxylin-eosin. Differential intracapillary leukocyte counts were obtained at a magnification of ×1,000. In each section, 10 different microscopic fields selected at random were examined, and the density of leukocytes was expressed as numbers per single alveolus. The same sections served as samples for estimating cell differentials. Mononuclear cells in the circulation were distinguished as being inside the endothelium, whereas macrophages were outside the endothelium. We identified the capillary endothelium based on its location surrounding alveoli.

Distribution of ICAM-1 in pulmonary microvessels. Fluorescence-labeled 1A29 (4 μg/g body wt) was injected into an anesthetized ventilated rat via the femoral vein. Five minutes after the injection of fluorescence-labeled 1A29, the rat was prepared for perfusion. Nonbinding fluorescence-labeled 1A29 was washed out of the perfused rat lung. ICAM-1 labeled with fluorescence-labeled 1A29 was visualized with a confocal laser scanning microscope assisted by the image analyzer mentioned above. To confirm anatomic orientation of the pulmonary microvasculature, the proximal and distal landmarks such as arterioles and venules were recognized by injecting FITC-labeled red blood cells or dextran at the end of each experiment.

We also examined the ICAM-1 distribution using a conventional immunohistochecmical approach. The lungs were fixed by intratracheal instillation of periodate-lysine-paraformaldehyde solution and embedded in optimal cutting temperature (OCT) compound (Miles, Elkhart, IN), then frozen in dry ice and acetone. Cryostat sections (6 μm each) were air dried for 1 h at room temperature. The sections were washed in PBS and incubated with 10% normal goat serum in PBS. To inhibit endogenous peroxidase activity, the sections were treated with methanol and 3% hydrogen peroxide for 20 min according to the method of Streefkerk (23). 1A29 antibodies were diluted 100-fold with PBS and absorbed with normal rat serum. Nonimmunized mouse serum was used instead of 1A29 as a negative control. These sera were layered on the section for 2 h at room temperature. Sections were incubated with peroxidase-labeled goat anti-mouse IgG (H+L) (Zymed, San Francisco, CA) for 30 min at room temperature and rinsed in PBS for 5 min three times. Then, labeled peroxidase was detected by reaction with 3,3′-diaminobenzidine tetrahydrochloride in 3% hydrogen peroxide/0.1% (hydroxyethyl)amino-
control, rat IL-8, anti-CD18, and anti-ICAM-1 groups are shown in Fig. 2. There were no differences in mean arteriolar \( V_r \) among the groups. Mean \( V_r \) in capillaries and venules were also comparable among groups.

### Table 1. Shear rates of pulmonary arterioles and venules

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<thead>
<tr>
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<th>Shear Rate, s(^{-1})</th>
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<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Arterioles</td>
<td>456 ± 66</td>
</tr>
<tr>
<td>Venules</td>
<td>552 ± 73</td>
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</table>

Values are means ± SE; control, rat interleukin-8 (IL-8), and anti-CD18 groups were each \( n = 6 \), and anti-intercellular adhesion molecule-1 (ICAM-1) group was \( n = 4 \). Mean blood cell velocity \( (V_{mean}) \) was estimated from centerline blood flow velocity \( (V_c) \). \( V_c \) was determined for arterioles and venules in all groups. \( V_{mean} \) was calculated from the following formula: \( V_{mean} = V_c / 1.6 \). Vessel diameters \( (D_v) \) of arterioles and venules were measured with the NIH image from the confocal laser scanning images. Vessel wall shear rate \( (\gamma) \) was calculated based on definition for a Newtonian fluid: \( \gamma = 8 (V_{mean}/D_v) \) (s\(^{-1}\)). There were no significant differences in arteriolar and venular wall shear rates among groups.

It should be noted that leukocytes displaying a “caterpillar” movement were unable to be observed either in arterioles or in venules of the pulmonary vasculature in any groups. When the rolling cells were defined as those moving more slowly than the “critical” velocity (12, 14), the rolling fractions of arterioles and venules of the normal group were 25.8 ± 5.5 and 19.0 ± 1.8% (SD), respectively. CINC/gro increased these fractions of arterioles (34.0 ± 5.7%) and venules (49.9 ± 10.0%). Mean \( V_w \) of pulmonary arterioles, capillaries, and venules in the control group were 1.43 ± 0.28, 0.64 ± 0.12, and 1.74 ± 0.42 mm/s, respectively. The treatment of CFSE-labeled leukocytes and the perfused rat lung with 2H5 did not affect velocities in pulmonary arterioles, capillaries, or venules (1.32 ± 0.44, 0.64 ± 0.11, and 1.75 ± 0.32 mm/s, respectively). Stationary adherent leukocytes were rarely observed in pulmonary arterioles and venules, and a majority of leuko-

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**Fig. 1.** Light microscopic view of rat alveoli (A), confocal laser scanning microscopic view of capillary networks (B), and fluorescein isothiocyanate (FITC)-labeled erythrocytes in vasculature (C). Same site is shown. Capillary networks were visualized by confocal laser scanning microscopy. Vessel diameters were measured utilizing NIH image. \( \text{v} \), venule; \( \text{c} \), capillary; \( \text{al} \), alveolus; arrows, erythrocytes. Velocities of leukocytes and erythrocytes were analyzed at a rate of 250 frames/s with high-speed video system. Bar = 20 µm.

**Fig. 2.** Mean red blood cell (RBC) velocities \( (V_r) \) in arterioles, capillaries, and venules of all groups. Open, solid, hatched, and striped bars denote control, rat interleukin-8 (IL-8), anti-CD18, and anti-intercellular adhesion molecule-1 (ICAM-1) groups, respectively. There were no \( V_r \) differences in arterioles, capillaries, or venules among groups. Values are means ± SE. Control, rat IL-8, and anti-CD18 groups were \( n = 6 \), and anti-ICAM-1 group was \( n = 4 \).
lytes was captured in capillaries. Some leukocytes exhibited transient arrest inside capillaries, whereas others suddenly stopped at the branching points and bifurcations of capillaries (Fig. 3). When we observed CFSE-labeled leukocytes under low-power magnification, some leukocytes treated with CINC/gro traveled through the capillary bed and into venules. Representative motion patterns of leukocytes in the control and the rat IL-8 groups are shown in Fig. 4. There were two distinct patterns of leukocyte behavior in capillaries of the control preparation. The majority of leukocytes were in “continuous” motion, with relatively little variation in velocity. A small number, however, stopped moving transiently (0.04–0.2 s) and then resumed moving within the capillary.

The latter “discontinuous” motion pattern, in which leukocytes stopped moving at least once for more than 0.04 s during observation of a 200 µm × 200 µm area of the lung periphery, occurred at a frequency of 21.2 ± 6.1% of the total number of leukocytes observed in the control group (Table 2). When leukocytes were pre-treated with CINC/gro, 47.3 ± 8.2% of the total number showed the discontinuous motion pattern within the capillaries of the aforementioned area. The CINC/gro-induced increase in the population of leukocytes showing the discontinuous motion pattern was attenuated when the lung and leukocytes were pretreated with MAbs against ICAM-1 and CD18, respectively.

The majority of control leukocytes moved across the aforementioned field within 0.3 s, but a small percentage of the cells took ~0.6 s. On the other hand, when prestimulated with CINC/gro, it took >0.6 s for leukocytes to disappear from the field, suggesting prolongation of leukocyte transit time due to the chemokine stimulation. Clearly, we observed numerous stationary leukocytes trapped in alveolar capillaries under these circumstances.

Fig. 3. Representative patterns of leukocyte entrapment in pulmonary capillaries. Intracapillary leukocytes were analyzed with confocal laser scanning microscopy at a rate of 1/250 s. A leukocyte stopping along a capillary wall (A) and a leukocyte plugging a capillary bifurcation (B) are shown. Arrows indicate leukocytes. Bar = 5 µm.

Fig. 4. Velocity analysis of leukocyte movement at each time point. Transverse axis indicates time from leukocyte appearance to disappearance, and longitudinal axis indicates leukocyte velocity at each time point. Representative movement patterns of leukocytes in control (A) and rat IL-8 (B) groups are shown. There were two main patterns of leukocyte movement in capillaries: a continuous pattern, in which leukocytes moved smoothly, and a discontinuous pattern, in which leukocytes repeatedly stopped moving. • and ■, continuous patterns, respectively, in the control group (A); ○, □, △: discontinuous patterns of three different leukocytes in rat IL-8 group (B).
There were no differences in the relative leukocyte velocities \( V_w/V_r \) in arterioles among the groups (Fig. 5). On the other hand, the \( V_w/V_r \) values in capillaries and postcapillary venules of the rat IL-8 group were decreased as compared with those of the control group. The capillary \( V_w/V_r \) values in the anti-CD18 and anti-ICAM-1 groups were restored to the control level. In postcapillary venules, the CINC/gro-induced reduction in \( V_w/V_r \) values was attenuated in part by anti-CD18 MAb and was abolished by anti-ICAM-1 MAb. The \( V_w/V_r \) values in arterioles, capillaries, and venules of the control for the anti-CD18 and anti-ICAM-1 groups were 0.97 ± 0.12, 0.39 ± 0.14, and 0.75 ± 0.11, respectively. These values were not different from those in arterioles, capillaries, and venules of the rat IL-8 group (0.82 ± 0.10, 0.46 ± 0.11, and 0.65 ± 0.11, respectively).

Histological examination of leukocyte sequestration in pulmonary capillaries. The number of leukocytes, including polymorphonuclear neutrophils (PMNs) and mononuclear cells, in the periphery of the perfused lung in the rat IL-8 group was 2.70 ± 0.50/alveolus, which was increased as compared with that of the control group (0.35 ± 0.09/alveolus) (Table 3). The increased leukocyte numbers in the rat IL-8 group were partly attenuated by treatment with anti-CD18 and anti-ICAM-1 MAbs, whereas those in the anti-CD18 (0.91 ± 0.17/alveolus) and anti-ICAM-1 (1.20 ± 0.08/alveolus) groups were significantly elevated as compared with that in the control group. The differential ratios of capillary leukocytes are shown in Table 3. CINC/gro increased the proportion of PMNs sequestered in capillaries by 19.2 ± 3.4-fold as compared with that of the control group.

The intracapillary leukocyte count in the sections of 2H5-treated perfused lung was 0.34 ± 0.06, a value not significantly different from that of the normal control. The sequestered leukocyte number of the control for the anti-CD18 and anti-ICAM-1 groups using isotype-matched mouse IgG instead of anti-CD18 or anti-ICAM-1 MAbs was 2.70 ± 0.86 counts/alveolus (PMN, 74.5 ± 5.5%; mononuclear, 25.5 ± 5.5%), which was not different from that of the rat IL-8 group (Table 3).

Distribution of ICAM-1 in pulmonary microcirculation. The ICAM-1 distribution, as revealed by fluorescence-labeled 1A29 and the confocal laser scanning microscope, was observed mainly in capillary networks (Fig. 6A) and venules (Fig. 6B). There was only slight expression of ICAM-1 in arterioles. The arterioles and venules were confirmed by FITC-dextran and FITC-labeled red blood cells (Fig. 6C). There was no detection of ICAM-1 in alveolar septums. The sequestration of leukocytes in pulmonary capillaries was increased as compared with that in control group. Increased leukocyte numbers in rat IL-8 group were attenuated by treatment with anti-CD18 and anti-ICAM-1 monoclonal antibodies. Leukocyte numbers in anti-CD18 and anti-ICAM-1 groups were, however, elevated as compared with that in control group. CINC/gro significantly increased proportion of polymorphonuclear neutrophils (PMNs) in pulmonary capillaries. *P < 0.05 and †P < 0.01 as compared with control group.

Table 2. Percentages of continuous and discontinuous leukocyte motion patterns in pulmonary capillaries

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Rat IL-8</th>
<th>Anti-CD18</th>
<th>Anti-ICAM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discontinuous, %</td>
<td>21.2±6.1 47.3±8.2* 24.8±4.8†</td>
<td>20.8±7.3‡</td>
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<tr>
<td>Continuous, %</td>
<td>78.8±6.1 52.7±8.2* 75.2±4.8‡</td>
<td>79.2±7.3‡</td>
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Values are means ± SE; control, rat IL-8, and anti-CD18 groups were each \( n = 6 \), and anti-ICAM-1 group was \( n = 4 \). Discontinuous pattern is defined as leukocyte motion in which transient cessation of movement occurred at least once for more than 0.04 s during observation of a 200 µm × 200 µm area of lung periphery. Discontinuous motion pattern was significantly more frequent in rat IL-8 group. Cytokine-induced neutrophil chemoattractant (CINC/gro)-induced increase in population of leukocytes showing discontinuous movement pattern was attenuated when lung and leukocytes were pretreated with monoclonal antibodies against ICAM-1 and CD18, respectively. *P < 0.01 as compared with control group. †P < 0.01 as compared with rat IL-8 group.

Table 3. Pulmonary capillary leukocyte numbers and differentials

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Rat IL-8</th>
<th>Anti-CD18</th>
<th>Anti-ICAM-1</th>
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<tbody>
<tr>
<td>Total number, counts/alveolus</td>
<td>0.35±0.09</td>
<td>2.70±0.50†</td>
<td>0.91±0.17‡</td>
<td>1.20±0.08‡</td>
</tr>
<tr>
<td>PMN, %</td>
<td>26.7±10.6</td>
<td>68.2±5.5‡</td>
<td>79.8±6.6†</td>
<td>70.8±5.0†</td>
</tr>
<tr>
<td>Mononuclear, %</td>
<td>72.9±10.7</td>
<td>31.8±5.5†</td>
<td>20.0±8.6†</td>
<td>28.8±4.9†</td>
</tr>
</tbody>
</table>

Values are means ± SE; control, rat IL-8, and anti-CD18 groups were each \( n = 6 \), and anti-ICAM-1 group was \( n = 4 \). Total leukocyte numbers and differential percentages within pulmonary capillaries in sections stained with hematoxylin-eosin are shown. Number of leukocytes per alveolus in perfused rat lung in rat IL-8 group was increased as compared with that in control group. Increased leukocyte numbers in rat IL-8 group were attenuated by treatment with anti-CD18 and anti-ICAM-1 monoclonal antibodies. Leukocyte numbers in anti-CD18 and anti-ICAM-1 groups were, however, elevated as compared with that in control group. CINC/gro significantly increased proportion of polymorphonuclear neutrophils (PMNs) in pulmonary capillaries. *P < 0.05 and †P < 0.01 as compared with control group.
able fluorescence along vessels in the lung pretreated with FITC-labeled mouse IgG (Fig. 6D).

We also examined the ICAM-1 distribution immunohistochemically (Fig. 7). The ICAM-1 was distributed not only on alveolar epithelial but also on endothelial cells, especially in venules and capillaries. There was very little ICAM-1 on pulmonary arterioles (Fig. 7).

Expression of adhesion molecules on leukocytes. Rat leukocytes were stimulated with 10 nM CINC/gro for 10 min to examine the surface expressions of SLeX, L-selectin, and CD18 on neutrophils and lymphocytes with a FACScan flow cytometry system (Fig. 8). In response to CINC/gro, L-selectin and SLeX were markedly downregulated, whereas CD18 was inversely upregulated on the surface of neutrophils. We confirmed that these changes elicited by CINC/gro occurred specifically on rat neutrophils but not on lymphocytes, supporting previous observations (18). These results suggest that, under the current experimental conditions, CINC/gro specifically stimulates neutrophils.

Leukocyte behavior in mesenteric microcirculation. In the mesentery, leukocytes exhibited characteristics different from those observed in the lung preparation. In the observed mesenteric microcirculation, the venular wall shear rates were between 300 and 500 s⁻¹, values no greater than those measured in the perfused lung preparation. Figure 9 illustrates time history of the influx of CFSE-labeled leukocytes in the mesenteric venules, showing no significant difference between the two groups until the initial 10-min recording period after the cell injection. However, the difference in the cell influx became evident in a time-dependent manner, and the disappearance rate of the CINC/gro-prestimulated leukocytes turned out to be greater than that of...
the unstimulated leukocytes, presumably because of entrapment of these prestimulated cells in other organs (e.g., lung).

Although the regional influx of the CINC/gro-prestimulated cells was almost equivalent to that of the cells unstimulated, the behavior of these cells in the venules exhibited quite distinct pictures between the two groups. Among these cells, ~30% of the unstimulated cells exhibited a typical caterpillar-like behavior, indicating ~0.2 in the $V_w/V_r$ value as seen in Fig. 10, top panel. On the other hand, the rolling flux observed in the CINC/gro-unstimulated cells was significantly diminished when the cells were prestimulated with CINC/gro. Under these circumstances, a major population of the cells displayed free-flowing behavior, showing ~0.6 in the $V_w/V_r$ values. These results indicate that CINC/gro pretreatment induces marked reduction of the baseline rolling of leukocytes in the mesenteric venules, illustrating different pictures from those observed in the pulmonary microcirculation.

Differences in the behavior of the CFSE-labeled leukocytes became even more evident, when the mesentery was superfused with proadhesive stimuli such as FMLP at 100 nM for 10 min. The unstimulated CFSE-labeled cells adhered to the venule at a density of $3.9 \pm 0.8$ cells/100-µm venular segment. In contrast, the CINC/gro-prestimulated cells did not exhibit any detectable adhesion ($0.5 \pm 0.3$ cells/100-µm venular segment). Such an adhesive response of CFSE-labeled cells did not occur in the absence of FMLP; the densities of adhesion in the CINC/gro-unstimulated and -prestimulated cells were $0.3 \pm 0.3$ and $0.4 \pm 0.3$ cells/100-µm venular segment without superfusion of FMLP, respectively. With the data shown in Fig. 8 taken into account, these findings suggest that, in the mesenteric...
between the 2 groups (signed ranking test, there was a significant difference in mean value in injection from 6 separate experiments. On the basis of Wilcoxon's were collected during 10-min observation period after completing cell and CINC/gro(1) indicate data collected from animals injected with CINC/gro-unstimulated and -prestimulated cells, respectively. Data were collected during 10-min observation period after completing cell injection from 6 separate experiments. On the basis of Wilcoxon's signed ranking test, there was a significant difference in mean value between the 2 groups (P < 0.05).

Discussion

The sequential multistep processes involving selectin-dependent rolling and integrin-dependent adhesion have been thought to serve as a central mechanism for stationary leukocyte interaction with endothelium in vivo and in vitro (6, 15, 22, 30). However, the present study first provides visible evidence showing specific patterns of interactions with microvascular endothelium including sudden arrest, plugging, and adhesion to capillary walls. In addition, the CD18-ICAM-1 interaction is likely to be attributable to the capillary entrapment, as much as immunoneutralization of these molecules attenuated the density of leukocytes trapped in the alveolar capillaries. As was observed in mouse lungs (5), ICAM-1 was shown to be expressed abundantly in rat alveolar capillaries. The CINC/gro-stimulated neutrophils are thus in a position to utilize their upregulated CD18 molecules to facilitate specific binding to capillary endothelial ICAM-1.

On the other hand, another mechanism to be taken into account is capillary entrapment through CD18-ICAM-1 independent mechanisms, since anti-CD18 and anti-ICAM-1 MAbs attenuated only a portion of the CINC/gro-induced capillary leukocyte entrapment. Changes in leukocyte deformability induced by activation and mismatch of leukocyte-capillary diameters have been postulated to be the mechanism underlying CD18-independent leukocyte sequestration in pulmonary capillaries (9, 34). Because IL-8 is known to markedly change the intracellular actin polymerization in neutrophils and thereby increase cell stiffness (33), changes in viscoelastic properties of the neutrophil membrane may serve as a mechanism mediating capillary leukocyte sequestration. Collectively, our observations showing mechanical entrapment of activated leukocytes in pulmonary microvessels (Fig. 3) provide evidence that an alternative mechanism independent of adhesion molecules underlies pulmonary leukocyte accumulation.

Doerschuk (8) reported that CD18 does not mediate the initial sequestration of neutrophils but is required to keep the sequestered neutrophils in the lungs for more than a few minutes. These results are consistent with ours in view of the fact that tissue accumulation of leukocytes in the lungs involves both CD18-dependent and -independent mechanisms, although there are several differences in the experimental protocols. Our observation was achieved by injecting a controlled number of CINC/gro-prestimulated leukocytes into the normoperfused lung. Accordingly, we could not observe the initial sequestration of neutrophils in the lung that is generally observed in response to the intravascular administration of stimuli such as zymosan-activated plasma into rabbits (8).
The capillary accumulation of stimulated leukocytes that occurs under physiological shear rates appears to be unique to the pulmonary microcirculation. In other organs such as the heart, brain, and skeletal muscles, the leukocyte entrapment in capillaries occurs only when the microvascular system is exposed to low-shear conditions elicited by hemorrhagic shock or ischemia (3, 7, 24). In addition, the current study has clinically important implications for understanding why the lung is a primary target of organ damage in endotoxemia and sepsis, in which a variety of cytokines including IL-8 are known to be released into the circulation (19, 21) and circulating neutrophils shed their L-selectin (21). Under these circumstances, such intravascular activation of neutrophils may cause redistribution of neutrophils in the marginating pool, i.e., demargination under disease conditions has yet to be elucidated, the present results shed light on the effectiveness and limitations of antiadhesion therapy, which may induce leukocyte accumulation and subsequent lung injury.

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Address for reprint requests: K. Yamaguchi, Dept. of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160 Japan.

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