Distribution and Immune Responses Resulting from Oral Administration of \( \text{d}-\text{Limonene} \) in Rats

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Summary We investigated the relationship between the absorptive pathway and the immune responses of the lung, particularly the phagocytic function of alveolar macrophages (Mφ) after oral administration of \( \text{d}-\text{limonene} \) in rats. \( \text{d}-\text{Limonene} \) was orally administered in oily solution with a stomach tube in thoracic duct-cannulated rats, and the lymphatic output of \( \text{d}-\text{limonene} \) was measured. \( \text{d}-\text{Limonene} \) levels reached a maximum in thoracic duct lymph and lung 3 h after its oral administration. It also significantly increased in bronchoalveolar lavage fluid (BALF) and alveolar Mφ, in which there was frequently a focal exudation of lipid droplets containing \( \text{d}-\text{limonene} \) into the alveolar cavity through alveolar capillary walls. Second, \( \text{d}-\text{limonene} \) orally given to rats (250, 500, 1,000 mg/kg/d) for 8 consecutive days resulted in a marked increase in both the number and the phagocytic activity of alveolar Mφ compared to the controls. BALF from rats dosed with \( \text{d}-\text{limonene} \) (1,000 mg/kg/d) enhanced the phagocytic activity of alveolar Mφ from control rats because the dose was prolonged. The activity of alveolar Mφ following in vitro incubation with \( \text{d}-\text{limonene} \) also increased in a dose-dependent manner. An oral administration of \( \text{d}-\text{limonene} \) enhanced the Con A-stimulated proliferation of splenocytes. These results suggest that \( \text{d}-\text{limonene} \) taken up from the thoracic duct lymph moves to the lung and directly activates the immune response of alveolar Mφ there, or indirectly activates it through activated lymphocytes.

Key Words \( \text{d}-\text{limonene} \), thoracic duct, lung, alveolar macrophages, phagocytosis

\( \text{d}-\text{Limonene} \) (\( \text{p}-\text{mentha-1,8-diene} \)) is a major component in the essential oils of citrus fruits and is widely used for the flavorings of food products, soft drinks, and juices. Furthermore, it has been associated with diverse biological activity spectra, such as antitumor activity against rodent mammary or lung cancer when fed during the initiation phase (1–6), dissolution of human gallstones (7), and modulatory immune response (8–11). Monoterpene including \( \text{d}-\text{limonene} \) enhances the percutaneous permeability to relate the absorption of some drugs (12, 13). It is suggested that \( \text{d}-\text{limonene} \) is absorbed from the intestine and transported mainly through thoracic duct lymph. Then it primarily reaches the lung and may directly affect lung tissues and alveolar macrophages (Mφ). On the other hand, there are some reports that terpenoids including \( \text{d}-\text{limonene} \) may irritate the mucous membrane and the skin (14), and further prolonged exposure of \( \text{d}-\text{limonene} \) may result in an impairment of lung function (15–18) and kidney (19–23).

Despite the great interest in lipid-soluble dietary factors, little is known about the role of \( \text{d}-\text{limonene} \) in immunoregulatory functions, particularly in the lung, and the relationship between its absorption process and the function of alveolar Mφ. The main purpose of the present study is to verify transport via thoracic duct, occurrence, and immune response in the lung after the oral administration of \( \text{d}-\text{limonene} \) to rats.

MATERIALS AND METHODS

Animals and treatments. Specific pathogen-free male Fisher rats (Japan SLC, Shizuoka) weighing 200–220 g were used. All rats were given a commercial laboratory diet (MF; Oriental Yeast Co., Tokyo) and water and housed individually in an air-conditioned room at 21 ± 2°C during the experiment. \( \text{d}-\text{Limonene} \) was obtained from Wako Pure Chemical Industries (Osaka, Japan) and dissolved in soybean oil. The rats were randomly divided into two groups of 4 rats each. The experimental group was given \( \text{d}-\text{limonene} \) (1,000 mg/kg) by means of stomach tube, except for an examination of the immune responses of alveolar Mφ and splenocytes after being given \( \text{d}-\text{limonene} \) (250, 500, or 1,000 mg/kg) for 8 consecutive days. The control group was given only soybean oil. This study was approved by the Ethical Committee of Tokushima University for Animal Studies.

Collection of samples. After a single oral administration of \( \text{d}-\text{limonene} \), the rats were anesthetized with sodium pentobarbital, and the thoracic duct was cannulated with silicon tube (0.5 mm outside diameter, Dow Corning Corp., Midland, MI, USA), according to

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the procedure of Bollman et al. (24). At 1, 3, 6, or 9 h of the experiment, lymph was collected in an ice-cold glass tube, and supernatant was then stored until analysis for D-limonene. Blood samples were drawn from the inferior vena cava and portal vein. After 1 h at room temperature, each serum was obtained by centrifugation. To collect bronchoalveolar lavage fluid (BALF) from the lung following the administration of D-limonene, the trachea was cannulated and the lungs were washed with physiological saline. Second, to obtain alveolar Mϕ from the lung of rat following the oral administration of D-limonene for 8 consecutive days, a similar washing of the lung was done as described above. BALF and alveolar Mϕ were then separated by centrifugation (400×g, 5 min, 4ºC). The lung and liver tissues were rinsed with physiological saline and minced with scissors. After centrifugation, the tissues were stored until the detection of D-limonene.

Gas chromatography-mass spectrometry (GC/MS). D-Limonene contents in the samples described above were analyzed by the GC/MS system (Hewlett-Packard 6890) by the use of a DB-17 capillary column (30 m×0.25 mm i.d.×0.25 μm film thickness). Helium was used as a carrier gas. D-Limonene in each sample was extracted by hexane and centrifuged at 1,650×g for 10 min at 4ºC. The hexane layer was injected into the column. The temperatures of the injection port and ion source were set at 250 and 280ºC respectively. The temperature of the oven was programmed to 50ºC for 4 min and increased to 150ºC at a rate of 6ºC/min.

Light microscopy. The lungs of rats 3 h after the oral administration of D-limonene were fixed in 10% formaldehyde solution at room temperature and embedded in paraffin. Lung slices 7 μm thick were stained with HE, and frozen sections of fixed tissues were stained with oil red O.

Preparation of macrophage-activating factor (MAF). Spleens from untreated rats were aseptically removed and minced. Splenocytes were then passed through a stainless steel mesh in medium. They were adjusted at 1×10⁷/mL and incubated with 5 μg/mL Con A, penicillin (100 units/mL), and streptomycin (50 μg/mL) for 6 h at 37ºC. After incubation, the supernatants were filtered and stored at −40ºC.

Preparation of alveolar Mϕ and BALF. The rats were anesthetized and exsanguinated by cutting off the arteries of both kidneys. The trachea was cannulated, and the lungs were then washed with physiological saline at 37ºC. More than 95% of the lavaged cells were alveolar Mϕ as judged by nonspecific esterase staining. The viability of the alveolar Mϕ assessed by trypan-blue dye exclusion was also more than 95% in all samples.

Phagocytosis of alveolar Mϕ. Phagocytosis was determined by a modified method of Moriguchi et al. (25). Alveolar Mϕ were counted and plated (1.5×10⁵/well) with RPMI 1640 medium, including 10% FBS for 1 h at 37ºC. After incubation, the plates were washed to remove nonadherent cells and further incubated with medium, BALF (1/10 dilution) or MAF (1/100 dilution), for 6 h at 37ºC. Alveolar Mϕ was incubated with sheep red blood cells (SRBC, Nihon Biotest, Tokyo) that had been opsonized with rat anti-SRBC IgG for 90 min at 37ºC. After incubation, the culture plates were rinsed once with distilled water to lyse nonphagocytosed SRBC and washed with physiological saline. All remaining adherent cells were lysed by 0.1 N NaOH. The lysate including phagocytosed-SRBC in alveolar Mϕ, which represents red color, was determined by a microplate reader at 415 nm. The data were expressed as the phagocytic index, which was calculated by assigning a value of 1 to the absorbance of alveolar Mϕ from the control group and by comparing this to the absorbance of alveolar Mϕ from the group treated with D-limonene.

Proliferation of splenocytes. Spleens from rats treated with D-limonene for 8 consecutive days were aseptically removed and passed through a stainless steel mesh. Isolated splenocytes were counted, plated (1×10⁷/well) in 96 well microplates, and cultured with Con A (5 μg/mL) or LPS (10 μg/mL) for 48 h at 37ºC. The cultures were then pulsed with [³H]thymidine (specific activity 25 μCi/mmol, Amersham, England). After 24 h incubation, the plates were harvested on glass fiber filter. Radioactivity was counted by a liquid scintillation counter (Matrix 9600M, Packard, Japan). The data were expressed as a stimulation index, which was calculated by assigning a value of 1 to the radioactivity of splenic lymphocytes cultured with medium and comparing this to the radioactivity of splenic lymphocytes cultured with mitogens.

Statistical analysis. Values are means±SD (n=4). The statistical significance was analyzed by paired Student’s t-test or Duncan’s multiple-range test. The differences with p<0.05 were considered significant in all experiments.

RESULTS

Distribution of D-limonene after single oral dosage
Following the administration of D-limonene (1,000 mg/kg), the levels of D-limonene in thoracic duct lymph and lung tissue were peaked at 3 h and then decreased at 6–9 h (Table 1). Particularly, the concentration of D-limonene in the lung was approximately 6 times higher at 3 h than at 1 h. Lymph flow in the thoracic duct at 1 h after cannulation was slight, and it was then difficult to detect by 3 h. The concentration of D-limonene in portal vein blood, liver, and serum was peaked at 1 h, and it gradually decreased. D-Limonene in the BALF...
Table 1. D-limonene levels in the thoracic duct lymph fluid, lung, portal vein blood, liver, and serum after an oral administration of D-limonene.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>6</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thoracic duct</td>
<td>—</td>
<td>—</td>
<td>16.6±0.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.38±0.65&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.40±2.57&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lung</td>
<td>0.38±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.37±0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.93±3.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.89±1.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.26±0.35&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Portal vein</td>
<td>0.48±0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.14±0.41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.18±0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.96±0.53&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.73±0.57&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver</td>
<td>0.90±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.4±4.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.1±5.40&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.47±1.30&lt;sup&gt;ce&lt;/sup&gt;</td>
<td>5.14±1.52&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum</td>
<td>0.54±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.54±0.65&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.82±0.46&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.60±0.05&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.32±0.39&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data were expressed in µg/g (lung and liver) or µg/mL (thoracic duct, portal vein, and serum). Values marked with different superscript letters are significantly different at p<0.05.

also increased significantly at 3 h, but the level in alveolar Mφ slightly increased (data not shown). The lung was examined histologically (HE and Oil red O staining) to detect morphological findings in this stage. Figure 1 shows an appearance of light eosinophilic substances in alveolar cavities (A), and these substances are stained by oil red O (B). The photographs indicate the close contact with the capillary of alveolar septa. These findings are not detected in the lung of control group (C).

Immunological effect in rats fed D-limonene for 8 consecutive days

The number of alveolar Mφ in rats orally given 250 mg D-limonene/kg for 8 consecutive days was significantly greater than in the control group or in rats given 500–1,000 mg D-limonene/kg (data not shown). In rats dosed with D-limonene (1,000 mg/kg/d), the phagocytic activity of alveolar Mφ increased significantly over that of the control group (Fig. 2A). However, alveolar Mφ from the control group became more phagocytic following in vitro treatment with MAF for 6 h and showed 2.5 times the activity of alveolar Mφ cultured only with medium (Fig. 2B). In the higher D-limonene group (1,000 mg/kg/d), alveolar Mφ did not respond to MAF and showed no increase of phagocytic activity compared to that of alveolar Mφ cultured only with medium.

The effect of BALF in rats given D-limonene on phagocytosis of alveolar Mφ

The lavage fluid of the lung from rats fed a high D-limonene (1,000 mg/kg/d) for 5 or 8 consecutive days was enhanced in the phagocytic activity of alveolar Mφ compared to that of the control group, and showed a 30% increase of phagocytic activity compared to that of alveolar Mφ incubated only with medium (Fig. 3). With the lavage fluid from control rats, there was no significant effect on phagocytic activity of alveolar Mφ.

Phagocytosis of opsonized-SRBC by alveolar Mφ following in vitro incubation with D-limonene

To examine the direct effect of D-limonene on the phagocytosis of alveolar Mφ, in vitro incubation with various levels of D-limonene was undertaken. The most effective concentration of D-limonene was 0.1–10 µM, which was also 2.5 times higher than alveolar Mφ cultured only with medium (Fig. 4).
Number and mitogenic responses of splenocytes

The number of splenocytes in rats given d-limonene (500–1,000 mg/kg/d) for 8 consecutive days was greater than in the control group, but not significantly (Table 2). The mitogenic responses of splenocytes in rats given d-limonene (250–1,000 mg/kg/d) were especially increased by incubation with Con A, and they reached a maximum in rats given d-limonene (500 mg/kg/d), but were no greater at 1,000 mg/kg/d. The proliferative responses of splenocytes to LPS also were greater in rats given d-limonene (500 mg/kg/d) than in control rats, but the increases were not significant. The increases in responsiveness were not larger compared to those with Con A.

Discussion

At first, the early process of absorption and distribution of d-limonene after a relatively high dose (1,000 mg/kg) oral administration was investigated by the use of male Fisher rats. The d-limonene level in tho-

Table 2. Number and mitogenic response of splenocytes after the oral administration of d-limonene for 8 consecutive days.

<table>
<thead>
<tr>
<th>Doses of d-limonene (mg/kg/d)</th>
<th>No. of splenocytes (×10⁷ cells)</th>
<th>Con A (Stimulation index)</th>
<th>LPS (Stimulation index)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.46±0.27</td>
<td>87.4±38.0</td>
<td>11.1±9.01</td>
</tr>
<tr>
<td>250</td>
<td>2.05±0.50</td>
<td>117±42.4</td>
<td>11.8±6.69</td>
</tr>
<tr>
<td>500</td>
<td>2.13±0.64</td>
<td>143±36.3</td>
<td>14.0±6.89</td>
</tr>
<tr>
<td>1,000</td>
<td>2.24±0.40</td>
<td>127±52.3</td>
<td>11.1±6.66</td>
</tr>
</tbody>
</table>

Mitogenic response was obtained as a stimulation index.
raccic duct lymph peaked within 3 h and decreased 6 or 9 h after administration. The lung and BALF also showed the highest level of d-limonene. On the other hand, the d-limonene level in the portal vein peaked within 1 h, but the level was about half that in the thoracic duct lymph. These results suggest that d-limonene is absorbed mainly via the thoracic duct from the gastrointestinal tract during several hours, and reaches essentially the lung and partly, via portal venous blood, the liver.

Histological findings of lipid containing d-limonene in the lung demonstrated lipid (d-limonene) exudation into alveolar cavities through the capillary wall of alveolar septa. These findings were concomitant with biochemically increased levels of d-limonene in the BALF. As the mechanism of d-limonene exudate into the alveolar cavity, it has been considered because of the increase permeability of the alveolar capillary. The high solubility of d-limonene in soybean oil suggests that it is easily absorbed and transferred via a lymphatic pathway, and finally via the high permeability of the capillary wall's cell membrane. In relation to the capillary permeability of d-limonene, the exudation rate depends largely on tissue solubility and the partition coefficients between tissue/blood and blood/air (26). Therefore the partition coefficients are important for the prediction of toxic or pharmacokinetics. For example, Falk-Filipsson et al. (18) determined the in vitro blood/air partition coefficients of d-limonene and showed its easy dissolubility in oil and blood. The solubility of d-limonene, lipophilic compound, in blood and tissues suggests that its respiratory uptake or release is high. In fact, monoterpenes are partly excreted through the lung, which generates a violet-like odor of the exhaled air (27). Part of the redissolved monoterpenes is exhaled through the lungs and possibly excreted with the bile. In these experiments, the high affinity of d-limonene to lipophilic body compartments such as the capillary wall of alveolar septa may be easily responsible for the exudation of d-limonene into the alveolar cavity. Falk-Filipsson et al. also assumed by the measurement of the surface pressure of the electrical monolayer after nonionic surfactant was injected between the monolayers. The local irritating action of nonionic surfactants is formed to be parallel with their hemolytic activity (28). It is considered that the mechanism of exudation into the alveolar cavity through the capillary wall is similar to that of this hemolytic action.

In this study, the number of alveolar MΦ significantly increased in rats dosed d-limonene (250 mg/kg/d). The phagocytosis of opsonized-SRBC by alveolar MΦ also increased in a dose-dependent manner, whereas the phagocytic response of alveolar MΦ after incubation with MAF showed a decreasing response in rats dosed d-limonene (Fig. 2). This result resembles the previous report of supplemented high vitamin E diet in rats (29). As shown in Fig. 1, d-limonene increased the permeability of the alveolar wall and resulted in an exudation within the alveolar surfactant, which directly contacts to alveolar MΦ and activates their phagocytic functions. It is noticed that the level of d-limonene in BALF increased in this experiment (data not shown). The phagocytic activity of alveolar MΦ in normal rats increased after in vitro incubation with BALF or d-limonene (Figs. 3 and 4). These findings suggest that d-limonene directly enhances the function of alveolar MΦ. On the other hand, Evans et al. (8) reported that d-limonene supplementation significantly increased antibody responses in mice immunized by keyhole limpet hemocyanin (KLH). In the splenocyte-proliferative responses to mitogens (Con A or LPS), a more-suppressive effect occurred in mice after 8 wk, compared to 4 wk of d-limonene treatment. A histopathological examination of the spleen from d-limonene-treated mice showed that excessive cellular proliferation occurred in the secondary lymph nodule (B cell area), and extensive cellular was seen in the paracortical T cell areas. Schulz et al. (11) reported that the treatment of PHA-stimulated lymphocytes with 5 mM d-limonene suppressed the [3H]levalonate labeling of proteins to a degree that correlated with the level of DNA synthesis inhibition. Also in this experiment, the oral administration of d-limonene more increased in Con A-stimulated proliferation of splenocytes in a dose of 500 mg/kg/d than in one of 1,000 mg/kg/d (Table 2). These results indicate that the enhanced phagocytosis of alveolar MΦ in d-limonene-treated rats are due not only to the direct action of d-limonene, but also to activated lymphocytes.

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