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Rat Kupffer cells were observed and counted under a scanning electron microscope after a single dose of zymosan. The cell number increased after 24 h and reached 2 times the control after 72 h. The percentage of cells presenting numerous microvilli increased from 24% to 86% over the same period. Carbon clearance, a measure of phagocytic activity, decreased temporarily, regained control values after 12 h, and attained a 4-fold elevation at 72 h. Phagocytic activity paralleled Kupffer cell number, but increased more relatively. This result probably reflected activation of resident Kupffer cells as noted by numerous thick microvilli.

KEYWORDS: kupffer cell, zymosan, scanning electron microscopy, carbon clearance, phagocytosis

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SCANNING ELECTRON MICROSCOPIC ANALYSIS OF KUPFFER CELL PROLIFERATION AFTER ZYMOsan ADMINISTRATION

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Abstract. Rat Kupffer cells were observed and counted under a scanning electron microscope after a single dose of zymosan. The cell number increased after 24 h and reached 2 times the control after 72 h. The percentage of cells presenting numerous microvilli increased from 24% to 86% over the same period. Carbon clearance, a measure of phagocytic activity, decreased temporarily, regained control values after 12 h, and attained a 4-fold elevation at 72 h. Phagocytic activity paralleled Kupffer cell number, but increased more relatively. This result probably reflected activation of resident Kupffer cells as noted by numerous thick microvilli.

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Kupffer cells play a very important role in the phagocytosis of foreign substances. In the diseased liver, these members of the reticuloendothelial system (RES) are increased in number and phagocytic activity (1-3). Administration of stimulant agents such as zymosan, endotoxin and estradiol increases the phagocytic activity (4, 5). However, it is not clear whether the increased phagocytic activity is due to Kupffer cell proliferation or recruitment of quiescent cells. In an attempt to answer this question, rat Kupffer cells, stimulated by zymosan (6, 7), were observed and enumerated by scanning electron microscopy (SEM). The number of cells was compared with phagocytic activity.

MATERIALS AND METHODS

Zymosan administration. Zymosan A (Sigma Chemical Co., USA) was dispersed in physiological saline, heated in boiling water (5 min), and resuspended (8, 9). Male Sprague-Dawley rats weighing 180-250 g each were used. Forty mg Zymosan A in 10 ml saline/kg body weight was injected via the tail vein (10). Control values were obtained from rats immediately before treatment.

Microscopy. Original Zymosan A was coated with platinum-palladium in an ion coater (Eiko, type IB-3, Japan), and observed by SEM (JEOL, model U-3, Japan) at an accelerating
voltage of 15 kV.

Four or five rats were studied before and 2, 4, 8, 12, 24, 48, 72, and 96 h after zymosan administration. After ether anesthesia, 0.5 ml (500 units) heparin was injected into the heart to prevent coagulation. The liver was irrigated with 500 ml of Ringer solution. Perfusion-fixation was accomplished with 200 ml of 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) introduced through the thoracic aorta (150 mm Hg perfusion pressure). The liver was cut into small blocks which were further fixed overnight in the glutaraldehyde, stained by the revised tannin-oxmium method (11), dehydrated in a graded series of ethanol solutions, and finally dried by the critical point method using liquid carbon dioxide. The specimens were fractured under a stereomicroscope (12, 13), and thinly sputter-coated with platinum-palladium in the ion coater. The preparations were observed by SEM (Hitachi, model HFS-2, Japan or JEOL, models SEM-2, JSM-U3, Japan) at accelerating voltages of 15-20 kV. Kupffer cells were counted on more than 10 micrographs 90 × 120 pm (HFS-2, SEM-2) or 100 × 100 pm (JSM-U3) in size at 1,000 × magnification.

For light microscopy (LM), the perfusion-fixed livers were transferred to 10% formalin, embedded in paraffin, sectioned to 5 μm, and stained with hematoxylin-eosin.

Carbon clearance. Phagocytic activity of RES was measured by the carbon clearance method (14, 15) at the same times as for microscopy. India ink (Drafting ink, Pilot Co., Tokyo) containing 57 mg of carbon particles/ml was diluted ten fold in saline. Ten ml/kg body weight of the diluted ink was injected into the inguinal vein. Blood samples (0.03 ml) were obtained from the jugular vein before and 5, 10, 15, 20, and 25 min after the carbon injection. Carbon concentration was determined by measuring the absorbance of the samples diluted in 3 ml 0.1% Na2CO3 at 660 nm in an electrophotometer (Hitachi, Japan) (16). The carbon clearance rate constant, K, was defined as C = Coe-t (C, blood carbon concentration at time t; Co, carbon concentration at time 0), and used as a quantitative measure of phagocytic activity (14, 15). Carbon particle distribution throughout the body was ascertained macroscopically 30 min after the injection.

RESULTS

SEM of zymosan. The zymosan was composed of yeast cell walls approximately 4 μm in diameter (6) (Fig. 1).

Light microscopic evaluation. Hepatocytes did not present remarkable changes in any specimen. Mononuclear cell infiltration appeared 48 h after zymosan administration, and 50 μm diameter granulomatous foci consisting mainly of macrophages were noted. The foci were prominent at 72 h (Fig. 2).

SEM analysis of the liver. Before stimulant administration, Kupffer cells were uniformly spaced, and 18% of them presented numerous microvilli (Fig. 3; Table 1). Four h after zymosan administration, some Kupffer cells had phagocytized material 4 μm in diameter (Fig. 4). The number of Kupffer cells with numerous microvilli increased at 48 h to 60%, and at 72 h to 86% (Figs. 5, 6; Table 1). Kupffer cell aggregation was most extensive at 72 h (Fig. 5, 7), at which time there were numerous microvillus processes and filopodia (Fig. 5). Kupffer cell size did not change.

Kupffer cell count per field (0.01 mm2) was 5.3 ± 0.3 in the untreated rats. It increased to 6.0 ± 0.7 after 24 h (p<0.05) and 7.3 ± 1.6 after 48 h (p<0.05) A
maximum of $10.7 \pm 2.8$ was reached after 72 h ($p<0.01$) (Fig. 8).

*Phagocytic activity.* The carbon clearance rate was $0.048 \pm 0.018$ in the control rats. It decreased to half shortly after zymosan administration, regained the baseline rate at 12 h and reached a maximum ($0.196 \pm 0.033$) after 72 h (Fig. 9).
Fig. 3. Scanning electron micrograph of control rat. Kupffer cells (K) with sparse and short microvilli are scattered throughout the lobule.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Total number</th>
<th>With thick microvilli</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>17</td>
<td>3</td>
<td>17.6</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td>16</td>
<td>57.1*</td>
</tr>
<tr>
<td>4</td>
<td>26</td>
<td>5</td>
<td>19.2</td>
</tr>
<tr>
<td>8</td>
<td>25</td>
<td>6</td>
<td>24.0</td>
</tr>
<tr>
<td>12</td>
<td>11</td>
<td>5</td>
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<tr>
<td>48</td>
<td>30</td>
<td>18</td>
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<tr>
<td>72</td>
<td>42</td>
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</tr>
<tr>
<td>96</td>
<td>33</td>
<td>16</td>
<td>48.5</td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.01

The carbon clearance rate correlated with Kupffer cell count.

The injected carbon particles were distributed mainly in the liver, and moderately in the spleen; little was found in the lungs. Clearance of the carbon particles predominantly reflected phagocytic activity in the liver.

DISCUSSION

The present SEM observations demonstrated increased Kupffer cells in zymosan-treated rats (Fig. 8). Kupffer cell hyperplasia already has been reported
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Kupffer Cell Proliferation

Fig. 4. Scanning electron micrograph of Kupffer cell (K) 4 h after zymosan injection. A particle about 4 \( \mu m \) (arrow head) is observed in the cell. H, hepatocyte, S, sinusoid.

Fig. 5. Rat liver 72 h after zymosan injection. Many Kupffer cells form an aggregate. H, hepatocyte.

using LM and transmission electron microscopy, but quantitative enumeration has been lacking (5, 17-19). Though Kupffer cells are difficult to identify by
Fig. 6. A Kupffer cell (K) 72 h after zymosan injection. Note the numerous microvilli and filopodia of the Kupffer cell. The Kupffer cell is in contact with a lymphocyte (L). H, hepatocyte; S, sinusoid.

Fig. 7. Many Kupffer cells (arrow heads) aggregate and form a granulomatous focus 72 h after zymosan injection. H, hepatocyte; S, sinusoid.

LM, they are easily identified and counted by SEM (3, 20, 21). The Kupffer cell population increased and reached a zenith 72 h after zymosan
administration (Fig. 8). Phagocytic activity, as assessed by carbon clearance, also reached its peak after 72 h (Fig. 9). Additionally, both the number and thickness of microvilli varied in parallel with phagocytic activity (Table 1). Kupffer cells with numerous microvillous projections indicate vigorous phagocytic activity (21, 22). The cell count was double that of the control rats after 72 h, while carbon clearance increased 4-fold. The increased activity per cell may be explained by
enhanced activation of individual Kupffer cells and/or active participation of extrahepatic RES components.

Kupffer cell proliferation was observed after a 24 h delay, which may be the time necessary for new formation of Kupffer cells. Two reports present opposing conclusions about the origin of Kupffer cell population increases: one indicates that Kupffer cells replicate in the liver (9), and the other suggests that they migrate from other organs (23). Deimann et al. (24) demonstrated that glucan, isolated from zymosan, induced hemopoiesis in the adult rat liver. Kupffer cell aggregations observed as granulomatous foci (9, 25), which first appeared 48 h after zymosan administration (Fig. 2, 5, 7), could be the proliferating site.

Intravenous administration of india ink (carbon particles) or zymosan is followed by decreased number of platelets after 2 h (10). Circulating platelets play a role in particle clearance by attaching carbon to the villiform surface (26). In this study Kupffer cell number was not changed after 2 h, but the phagocytic index was decreased and Kupffer cells with numerous microvilli increased. Therefore, phagocytosis of the carbon particles probably was hindered by the zymosan administration. In Summary, phagocytic activity correlated with Kupffer cell population. Stimulated Kupffer cells presented numerous thick microvilli. These findings strongly suggest that phagocytic activity is related to the Kupffer cell's surface area.

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