Baseline Activity of Kupffer Cells Increases With Old Age

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Age-related changes in Kupffer cell numbers and function may have important implications for systemic immune responses and hepatic function. We compared numbers of Kupffer cells in the hepatic sinusoids and phagocytic function of Kupffer cells in isolated perfused livers of young, middle-aged, and old rats. On light microscopy, the number of Kupffer cells per 29,500 μm² field increased with increasing age (young 2.0 ± 0.2, n = 8; middle aged 3.3 ± 0.3, n = 7; old 5.5 ± 0.6, n = 7). After a single pass through the liver, the ratio of the fractional recovery of 500 nm polystyrene microspheres to that of sucrose decreased significantly with increasing age: young rats, 89 ± 35% (n = 7); middle-aged rats, 58 ± 18% (n = 9); and old rats, 49 ± 24% (n = 10), suggesting increased Kupffer cell phagocytic activity. In old age, increased Kupffer cell numbers and activity were observed in the basal state.

Old age is associated with decreased ability to withstand external stressors. Much of the age-related increase in susceptibility to substances that enter via the gastrointestinal tract has been attributed to the age-related decrease in hepatic clearance. The causes of decreased hepatic clearance with increasing age include the age-related reduction in liver mass and portal blood flow (1), reduction in transfer across the sinusoidal endothelium (2–5), and reduced phase I oxidative hepatic metabolism (6).

Age-related changes in the phagocytosis of xenobiotics and endobiotics in the portal blood, which occurs in the cells of the hepatic sinusoids, may also contribute to susceptibility to disease. Phagocytosis of smaller particles is performed by the hepatic sinusoidal endothelial cells (7–9). Endothelial scavenger receptors clear the blood of physiologic waste products such as oxidized low-density lipoproteins, matrix components, and advanced glycation end products from normal cell turnover processes (10,11). A recent study in mice demonstrated reduced scavenger function of the hepatic sinusoidal endothelial cells in old age (12).

Phagocytosis of larger particulates and foreign materials in the portal blood is performed by the Kupffer cells, which are endogenous liver macrophages that reside in the sinusoidal lumen (7,8,13). Substrates, including microorganisms, endotoxins, old and foreign cells, complement components, immune complexes, and collagen fragments, are phagocytosed by the Kupffer cells, and micro-organisms are killed by both oxygen-dependent and -independent mechanisms (14,15). Kupffer cells clear 80%–90% of injected particulate material (16). Although Kupffer cells have been observed in increased numbers in old age (17,18), the function of these cells in normal aging is not well established. The immune system undergoes complex and continuous remodeling with age (19), and functions of the innate immune system, including chemotaxis, phagocytosis, natural cytotoxicity, and complement activity, are relatively well preserved in elderly individuals (20). Aging is conceptualized as the result of chronic stress impinging on the macrophage, with a direct relationship between age and macrophage activation, mostly responsible for the presence of a subclinical chronic inflammatory process in elderly persons (21). Therefore, it is possible that the function of Kupffer cells, which account for 80%–90% of the body’s resident macrophages (22), is maintained or even increased in old age.

Previous studies have demonstrated that in the liver, particles < 100 nm pass through the fenestrations in the sinusoidal endothelium to enter the space of Disse (23–25), those 100–500 nm in diameter are phagocytosed by the sinusoidal endothelium, and particles > 500–1000 nm in diameter are taken up by Kupffer cells (7,8). Studies of liposomes have clarified that not only diameter, but also deformability and interaction with sinusoidal endothelial cells, influence the hepatic disposition of liposomes (26–28). In early studies, Wisse and colleagues (29) found that liposomes were primarily taken up by Kupffer cells. Perfusion of mouse livers with liposomes has shown that larger liposomes (500 nm) localize quickly and predominantly to Kupffer cells, whereas smaller liposomes (80 nm) localize to parenchymal cells, indicating that these smaller particles have passed through the endothelial fenestrations (30).

Any age-related changes in Kupffer cell number and function may have important implications for systemic immune responses as well as hepatic function. Therefore, in isolated perfused livers of young, middle-aged, and old rats, we compared numbers of Kupffer cells in the hepatic sinusoids and the hepatic volume of distribution in and hepatic extraction of 500 nm fluorescent polystyrene microspheres. Based on previous studies (24,28,30) and the theory of ultrafiltration [ ultrafiltration of particles through pores in synthetic membranes decreases when the radius of the particle exceeds 30 ± 5% of the radius of the
fluorescent microspheres (30–612 nm, respectively). The injectate contained 500 nm max 377, 517, and 588 nm; emission max 479, 546, and were stained with three different fluorescent dyes (excitation sciences, Warrington, PA) were 500 nm in diameter and liver. The polystyrene microspheres (Fluoresbrite; Poly- were fixed for light and electron microscopy. Morphometric dilution experiments were performed as described pre-

Methods

Animals
Young (aged 4–6 months, weight 300–400 g), middle-aged (aged 13–14 months, weight 380–440 g), and old (aged 24–26 months, weight 300–440 g) male Fischer 344 rats were imported from the National Institute on Aging (Bethesda, MD). All of the animals were specific pathogen free, housed in microisolator cages, and were allowed free access to water and sterilized standard commercial rat pellets (Harlan Global Rodent Diet; Harlan Teklad, Frederick, MD). Experiments were performed between 2 and 8 weeks after the rats arrived in Australia. The studies were approved by the Central Sydney Area Health Service and University of Sydney Animal Welfare Committees.

Perfusions and Multiple Indicator Dilution Experiments
Rats were anesthetized with pentobarbitone sodium (Rhone Merieux, Pinkenba, Australia). The portal vein and the thoracic inferior vena cava were cannulated. The livers of one cohort of rats (n = 8 young, n = 7 middle aged, and n = 7 old) were perfused with heparinized saline and fixed for light and electron microscopy. Morphometric analysis was performed as described previously (2).

In the second cohort of rats (n = 7 young, n = 9 middle aged, n = 10 old), liver perfusions and multiple indicator dilution experiments were performed as described previously (4). The perfusate was Krebs–Henseleit bicarbonate buffer at an approximate flow rate of 1 mL/min/g of wet liver. The polystyrene microspheres (Fluoresbrite; Polysciences, Warrington, PA) were 500 nm in diameter and were stained with three different fluorescent dyes (excitation max 377, 517, and 588 nm; emission max 479, 546, and 612 nm, respectively). The injectate contained 500 nm fluorescent microspheres (30 μL) and [14C]sucrose (1 μL), with total volume made up to 100 μL with Krebs–Henseleit buffer. Sucrose is a nonextracted substrate that travels unimpeded through the vascular and extracellular space and is not taken up by hepatocytes. After administration of each injectate, 30 outflow samples were collected at 2-second intervals. Hepatic viability was confirmed by macroscopic appearance, oxygen consumption, portal venous pressure, and electron microscopy. Outflow samples were analyzed for 14C specific activity (Packard Liquid Scintillation Counter; Sydney, Australia), and fluorescence was quantified with a Hitachi F-4010 fluorimeter (Hitachi Ltd., Tokyo, Japan).

Dose-normalized outflow time–activity curves were constructed as described previously (4). The mean transit time (MTT) was estimated from the ratio of the area under the first moment of the curve (AUMC) and area under the curve normalized for dose (AUC). MTT was corrected for the catheter and nonexchanging vessel transit time (k0), estimated from the time of first appearance of radioactivity above background levels. The volume of distribution (V) was determined from the product of the MTT and the flow rate (Q). Percentage recovery (R) of the injected microspheres and sucrose at 1 minute was calculated. Hepatic extraction (E) was calculated as 1 – R.

Microscopy
After completion of the perfusions (and the multiple indicator dilution experiments in the second cohort), liver specimens were fixed for light microscopy in 4% buffered paraformaldehyde and for electron microscopy with 2% glutaraldehyde/3% paraformaldehyde in 0.1 M sodium cacodylate buffer (0.1 M sucrose, 2 mM CaCl2). Light microscopy after hematoxylin and eosin staining was used to detect underlying disease, and specimens with liver pathology were excluded. Two old rats from the first cohort and one middle-aged and two old rats from the second cohort were excluded because of disseminated lymphoma. Livers of both cohorts of rats were also prepared and examined using transmission and scanning electron microscopy as described previously (2,4).

Statistics
Comparison of nonparametric morphometric data among young, middle-aged, and old rats was tested using the Kruskal–Wallis one-way analysis of variance. If significance was found, a Mann–Whitney U test was performed on the age groups to establish where the key significance lay. Comparison of the parametric pharmacokinetic data for young, middle-aged, and old rats was performed using t tests between each age group.

Results
Quantitation of Kupffer cells in livers from rats of different age groups was estimated with light microscopy analysis of hematoxylin and eosin–stained sections. The number of Kupffer cells per 29,500 μm2 field increased with increasing age (young 2.0 ± 0.2, n = 8; middle aged 3.3 ± 0.3, n = 7; old 5.5 ± 0.6, n = 7). Representative sections from young, middle-aged, and old rat livers are shown in Figure 1A–C, respectively. The increases in frequency of Kupffer cells that were observed between young and old rats and between middle-aged and old rats were statistically significant (p < .001, p < .01, respectively) and are shown in Figure 1D.

The fractional recovery of microspheres after a single pass through the liver was calculated using the multiple indicator dilution technique in the isolated perfused rat liver. The fractional recovery of microspheres after a single pass through the liver is the proportion of the microspheres that are injected into the portal vein that are collected from the inferior vena cava outflow tubing (i.e., the proportion not retained in the liver). These values were normalized to those calculated for sucrose, as sucrose is not extracted by the liver. After a single pass through the liver, the ratio of the fractional recovery of 500 nm microspheres compared to that of sucrose decreased significantly with increasing age: young rats, 89 ± 35%; middle-aged rats, 58 ± 18%;
and old rats, 49 ± 24%. With increased age, the decreased fractional recovery and therefore increased hepatic extraction suggests increased Kupffer cell phagocytic activity. Results are shown in Table 1.

From the results of the multiple indicator dilution technique experiments, estimates of the apparent volumes of distribution of microspheres and sucrose were calculated. The ratio of the apparent volume of distribution of 500 nm microspheres to that of sucrose was 0.62 ± 0.22 in livers of young rats, 0.52 ± 0.09 in the livers of middle-aged rats, and 0.61 ± 0.31 in livers of old rats (n = 7, n = 9, n = 10, not significant). In all age groups, the volume of distribution of microspheres is less than that of sucrose, the marker of the extracellular space (which includes both the vascular space and the space of Disse). This finding indicates that, in livers of young, middle-aged, and old rats, 500 nm microspheres are restricted to the vascular lumen and are unable to enter the space of Disse. However, the extraction of microspheres means that their apparent volumes of distribution are likely to be an underestimate.

Representative outflow curves for multiple indicator dilution experiments with microspheres and sucrose in livers of young, middle-aged, and old rats are shown in Figure 2. The microsphere outflow curves have a smaller AUC than do the sucrose outflow curves in the middle-aged and old livers, indicating that the hepatic extraction of microspheres (presumably by Kupffer cells) is greater in old age. In all age groups, the peaks in the microsphere outflow curves are narrower than those in the sucrose outflow curves, consistent with smaller volumes of distribution and significant hepatic extraction.

On electron microscopy, 500 nm microspheres were observed in the sinusoidal lumen and never in the extracellular space of Disse. Microspheres were seen inside vesicles of Kupffer cells, consistent with the assumption that microspheres of this diameter are extracted through uptake by Kupffer cells (Figure 3). In addition, the liver sinusoidal endothelial cells and space of Disse from the old rats had the morphologic changes of pseudocapillarization as described previously in old age (2,4).

### DISCUSSION

The observation of increased frequency of Kupffer cells with increasing age reported here is consistent with previous studies. Increased Kupffer cell numbers have been observed in the livers of older people in the absence of pathologic

<table>
<thead>
<tr>
<th>Age Group</th>
<th>% Recovery of Microspheres</th>
<th>% Recovery of Sucrose</th>
<th>Ratio of Recovery of Microspheres to Recovery of Sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young (n = 7)</td>
<td>80 ± 32</td>
<td>92 ± 12</td>
<td>89 ± 35</td>
</tr>
<tr>
<td>Middle aged (n = 9)</td>
<td>53 ± 14</td>
<td>94 ± 11</td>
<td>58 ± 18</td>
</tr>
<tr>
<td>Old (n = 10)</td>
<td>46 ± 21</td>
<td>94 ± 13</td>
<td>49 ± 24</td>
</tr>
</tbody>
</table>

Note: There is a significant decrease in the recovery of microspheres and in the ratio of recovery of microspheres to that of sucrose between young and middle aged and young and old age groups (p < .05).
causes (17,18,32). Martin and colleagues (33) reported a consistent volume density of Kupffer cells in female Sprague-Dawley rats aged 2 and 24–25 months but did not report cell numbers. Quantitation of Kupffer cells in our study was limited to light microscopy analysis of hematoxylin and eosin–stained sections, so it can only be considered an estimate. Even so, the increase in Kupffer cell numbers was nearly 3-fold in these rats and clearly consistent with a substantial aging change.

The significantly increased extraction of 500 nm microspheres by the livers of aged rats is most likely related to an increase in Kupffer cell phagocytosis in old age. It has been demonstrated previously that Kupffer cells phagocytose polystyrene microspheres 500 nm in diameter preferentially to smaller particles (34). We observed Kupffer cells in contact with large microspheres on electron microscopy and inside vesicles within the Kupffer cells. In light of the increased number of Kupffer cells observed in old age, it is not clear whether the increase in overall Kupffer cell function represents an increase in the phagocytic function of each cell. The magnitude of the increase in the number of Kupffer cells in old age (approximately 3-fold) is slightly less than the increase in the extraction of the microspheres (approximately 5-fold), suggesting that both increased numbers and activity of Kupffer cells may be implicated. Another possible explanation for the observed overall increase in basal Kupffer cell phagocytic activity with increasing age is that, in keeping with the mosaicism of immunosenescence (35), the activity of some subpopulations of Kupffer cells increases whereas that of others may decrease.

Thus this study establishes that basal phagocytosis by Kupffer cells is increased in old age. The finding is consistent with other observations of immunosenescence: The innate immune system is relatively spared, whereas the adaptive immune system declines (19,20), and it has been proposed that the chronic inflammatory state in aging is associated with macrophage activation (21). Previous studies used colloidal carbon uptake to assess the functional capacity of Kupffer cells in aging. In Kupffer cells stimulated by infusing colloidal carbon into perfused mouse livers for 15 minutes, Videla and colleagues (36) observed a decrease in carbon uptake and carbon-induced oxygen consumption in old age. Vomel and colleagues (37) found no change in carbon uptake with age in the isolated perfused rat liver. Yamano and colleagues (38) observed a non-significant increase in removal of colloidal carbon from the blood after 3 minutes in old rats compared with young. In a study measuring the phagocytic activity of young and old isolated perfused rat livers using a glycoprotein (Cucuruloplasmin), researchers found that the phagocytic activity of the old rat liver was significantly higher than that of the young rat liver (37). Interpretation of the results of these studies must consider the contribution of uptake of colloidal carbon by sinusoidal endothelial cells (39). Ito and colleagues (12) recently demonstrated reduced scavenger function by the sinusoidal endothelial cells with age. Phagocytosis of the 500 nm-diameter spheres used in our study is more specific for Kupffer cell uptake than is phagocytosis of colloidal carbon (30).

It is interesting that, whereas baseline Kupffer cell phagocytosis appears to increase with age, activation of Kupffer cells by administration of a hepatotoxic dose of cadmium occurs in young but not old rats (38). This finding may also explain why our study (which tested the phagocytic activity of predominantly inactivated Kupffer cells, using a single pass and injection of tracer amounts of microspheres) observed an increase in Kupffer cell phagocytosis, whereas previous studies [which examined the function of activated Kupffer cells after prolonged colloidal carbon infusion (36)] observed a reduction in Kupffer cell phagocytosis in old age. This reduced response to stimulation may be because Kupffer cells are chronically activated in old rat livers (21) or represent the lack of reserve in old age. In aging rats, increased basal activation of peritoneal macrophages, evidenced by increased basal expression of interleukin 6 (IL-6), and decreased response to stressors, such as decreased tumor necrosis factor-α (TNF-α) response to lipopolysaccharide, have been observed in vitro (40).

In old age we have found increased Kupffer cell numbers and activity in the basal state. Increased basal Kupffer cell

Figure 2. Representative outflow curves for 500 nm microspheres and sucrose in perfused livers from young (a), middle-aged (b), and old (c) rats. In comparison to the sucrose curve, the microsphere outflow curve has a smaller area under the curve in the old liver, indicating that the extraction of microspheres by Kupffer cells is greater in old age.
phagocytosis in old age may represent the chronic inflammation correlated with aging and possibly a response to increased antigen exposure. Even so, old age is associated with increased susceptibility to morbidity and mortality associated with portally delivered pathogens, indicating that these changes are not associated with improved clinical outcomes.

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References


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