Scintigraphic In Vivo Assessment of the Development of Pulmonary Intravascular Macrophages in Liver Disease*

Experimental Study in Rats With Biliary Cirrhosis

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Study objectives: In regard to nuclear medicine literature reporting lung uptake of colloidal radiopharmaceuticals in patients with liver diseases, it has been hypothesized that liver abnormalities could trigger induction of pulmonary intravascular macrophages (PIMs) in humans normally lacking them. Recently, experimental induction of PIMs in rats in which they are not normally prevalent has been demonstrated to be at the origin of pulmonary hemodynamic alterations with an increased susceptibility to ARDS. If such induction may occur in humans, the risk of pulmonary hemodynamic alterations has to be considered and detected. This study demonstrates in a rodent model of biliary cirrhosis that scintigraphy of phagocytic function as commonly used for liver exploration is a suitable strategy for staging PIM development.

Design: Sixty rats were randomized as follows: bile duct section (n = 40), sham operation (n = 10), and no operation (n = 10). The rats were submitted to scintigraphy of phagocytic function every 5 days over 35 days for the assessment of radiocolloid uptake within lung and liver. At day 35, radioactivity of blood was counted and immunohistochemistry was performed on lung specimens.

Results: As disease progressed, radiopharmaceutical uptake decreased within the liver, while increasing considerably in the lung. At day 35, lung uptake averaged about 66% as compared to 3% before surgery. Lung histologic findings revealed numerous intravascular mononuclear cells closely related to the monocyte-macrophage lineage.

Conclusion: Scintigraphy of phagocytic function commonly used for liver scanning could be a suitable strategy for the diagnosis of the induction of PIMs under pathologic situations.

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Key words: biliary cirrhosis; pulmonary intravascular macrophages; pulmonary phagocytosis; rat; scintigraphy

Abbreviations: PIM = pulmonary intravascular macrophage; RES = reticuloendothelial system

Pulmonary intravascular macrophages (PIMs) are large mononuclear cells different from interstitial and alveolar macrophages and are routinely found closely opposed to the endothelium of the pulmonary capillaries in several animal species, such as sheep, horse, goat, and pig.1–5 In these species, PIMs act as a part of the reticuloendothelial system (RES) in clearing particles from blood, such as bacteria, cellular debris, fibrin, colloids, and liposomes. Such phagocytic activity has been demonstrated6–8 to trigger the release of vasoactive mediators by PIMs, resulting in serious and potentially fatal pulmonary hemodynamic disturbances. Thus, this effect was observed after IV injection of any phagocytobable drugs (contrast agents, monastral blue, liposomes) and is independent of their intrinsic pharmacologic properties.9–12 In human and other species, such as rat, dog, and monkey, it is generally accepted11,12 that lung does not contain PIMs under normal conditions. It has therefore been postulated13–16 that phagocytic mononuclear cells may become adherent

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to the pulmonary endothelium under pathologic circumstances. Nuclear medicine literature has indeed reported many cases of lung uptake of colloidal radiopharmaceuticals in human patients with liver abnormalities.\textsuperscript{17-22} If such induction occurs in humans, the risk of pulmonary hemodynamic alterations has to be considered, as well as the clinical consequences of chronic secretion of proinflammatory mediators in the lung.

The purpose of our study was to define a noninvasive strategy suitable for \textit{in vivo} quantitative assessment and staging of the induction of phagocytic cells within the lung using a well-established model of PIM development in animals that do not normally have them: the rat with chronic biliary cirrhosis.\textsuperscript{23,24} We therefore chose a surgical induction as an alternative to the usual carbon tetrachloride model to avoid the direct toxic effect of this chemical agent on the pulmonary vessels. Sixty animals (40 animals undergoing operation and 20 control animals) were submitted to scintigraphic imaging of phagocytic function before surgery, and then every 5 days over 35 days. For all groups, lung and liver uptake of radio colloids was monitored as a function of time. At the end of the study, blood samples were taken and their radioactivity counted. Histologic examination and immunohistochemistry were used to characterize the cells induced within the pulmonary capillaries.

**Materials and Methods**

**Animals and Surgery**

For all surgery and imaging protocols, animals were anesthetized using ketamine, 80 mg/kg IM, and xylazine hydrochloride, 12 mg/kg IM. The study was conducted using 60 male Wistar rats weighing 250 to 300 g (Ifca Crede; Lyon, France). Experiments were performed according to the European guidelines for animal welfare. An initial scintigraphic examination was performed 2 days prior to surgery to establish baseline phagocytosis imaging status. This presurgical scintigraphy was performed according to the operating instructions described below. Animals were then randomly classified into three groups for surgery:

- **Group A, Common Bile Duct Ligation and Section** (n = 40): Laparatomy was performed, the common bile duct was isolated and double ligated, and a part of up to 5 mm was resected between the two ligatures.\textsuperscript{25} The abdominal incision was then closed with sutures, and the rats were allowed to recover.
- **Group B, Sham Surgery** (n = 10): The same surgical protocol as in group A was performed, but the common bile duct was neither ligated nor sectioned.
- **Group C, Control** (n = 10): No surgery was performed on these animals.

**Clinical State**

The clinical state of the animals was evaluated throughout the study to detect any signs of cirrhosis and ascites.

**Selection of a Radiopharmaceutical for the Imaging of the Phagocytic Function**

Our protocol required a radiopharmaceutical that provides, under physiologic situations, liver-uptake imaging reflecting phagocytic activity of the RES without any significant vascular contribution. This requirement led us to select large-sized \textsuperscript{99m}Tc tin colloids rather than \textsuperscript{99m}Tc sulfur colloid particles. Indeed, liver uptake of \textsuperscript{99m}Tc sulfur colloids has been documented\textsuperscript{25} to depend almost entirely on effective liver blood flow rather than on phagocytic activity of Kupffer cells. The particle size of colloidal radiopharmaceutical has also been demonstrated\textsuperscript{26-28} to determine organ distribution \textit{in vivo}, with bone marrow having a particular affinity for smaller particles (diameter < 150 nm). Thus, we optimized tin colloid formulation by selecting large particles ranging in size from 180 to 330 nm.

**Preparation of \textsuperscript{99m}Tc Tin Colloid Formulation:** Two formulations were prepared to optimize size distribution of particles. Batches containing 0.125 mg of stannous fluoride and 1.0 mg of sodium chloride were prepared without (batch A) or with addition (batch B) of nonionic surfactant polyethylene polypropylene glycol at 0.250 mg/mL of the final concentration. Aliquots were distributed in vials under nitrogen atmosphere and immediately frozen with liquid nitrogen, freeze-dried, and then sealed under vacuum to provide the colloid-labeling kit. For labeling, this kit was dissolved with 2 mL of isotonic saline solution and 555 mBq (15 mCi) of sodium \textsuperscript{99m}Tc pertechnetate (\textsuperscript{99m}TcO\textsubscript{4}-) freshly eluted from a \textsuperscript{99m}Tc generator (Eumimatic III; CIS Biointernational; Gif-sur-Yvette, France) was added; the preparation was then made up to a 5-mL final volume with saline solution. Analytical controls of radioactive solutions were performed to select the most suitable batch for \textit{in vivo} phagocytosis imaging. Controls consisted of radiocolloid high-performance liquid chromatography, nanosizing and \textit{in vivo} biodistribution in three additional healthy rats.

Exclusion-diffusion radioactive high-performance liquid chromatography (LB506, Berthold) was performed on a TSK 4000 SWXL column (Tosohas; Tokyo, Japan) using 1 mL/min 0.9% NaCl as eluent to determine radiochemical purity, \textit{i.e.}, to assess the content in labeled small-sized colloids and to assess free \textsuperscript{99m}Tc likely to alter the sensitivity and the specificity of imaging. Size determination of colloids was further determined using photon autocorrelation spectroscopy (N4MD Coulter Nanosizer; Coultronics; Havertown, PA), antimony sulfide colloids (Mallinckrodt; St. Louis, MO) being used as a reference to assess the accuracy of the sizing measurements. \textit{In vivo} biodistribution was performed using three additional healthy rats. These animals were injected IV via the penis vein with 27 mBq (1 mCi) of \textsuperscript{99m}Tc tin colloids. The animals were killed by a lethal dose of ketamine 30 min later. The lungs and livers were removed and their radioactivity counted (CRC-activimeter; Capintec; Ramsey, NJ).

**Scintigraphic Imaging and Blood Radioactivity Counting**

All the animals were anesthetized then injected IV via the penis vein with 37 mBq (1 mCi) of radiopharmaceutical corresponding to 8.3-\textmu g stannous colloids in 0.33-mL radioactive solution. Thirty minutes after injection, a 2-Min venital static image was acquired using a gamma camera equipped with a high-resolution, low-energy, parallel collimator (Orbiter 75; Siemens; Munich, Germany). Data were recorded using a 15% window centered on the 140-keV photopeak of \textsuperscript{99m}Tc into a 128 x 128 matrix on a dedicated computer system for digital display and analysis (Micropax II; Digital Equipment Corporation; Boston, MA).

The distribution of radioactive colloids was assessed on static images using regions of interest created for the liver, the lung,
and the whole body of the rat. The count rate recorded within these regions was used to calculate the percentage of liver and the percentage of lung uptake for each animal (ie, % liver = [total counts in liver/total counts in rat] × 100, and percent lung = [total counts in lung/total counts in rat] × 100). Liver and lung uptake was averaged within each group (A, B, and C) and plotted as a function of time.

After the last scintigraphy (day 35), blood samples were taken from three control rats and three cirrhotic rats 35 min after IV injection of radiopharmaceutical. Radioactivity of 1-mL blood samples was counted using a A500 CD Packard Gamma Counter (Hewlett-Packard; Palo Alto, CA). The total blood activity was determined from the theoretical blood volume estimated to 6% of the body weight. Results were expressed as the percentage of the injected radioactive dose.

Statistical Analysis

Comparison between the groups was performed using Student’s t test (two-tailed).

Histology

Histologic examination was performed on liver and lung at the end of the study, (ie, 35 days after surgery) to confirm the cirrhosis in the liver and the appearance of mononuclear cells within the lung capillaries. Nine animals (three animals of each group) received a lethal dose of ketamine, and the lung and liver were removed and fixed in 10% neutral formalin. All the specimens were then embedded in paraffin and 5-μm axial sections were obtained. The lung and liver specimens were stained using both Martius Scarlet Blue and hematoxylin-eosin.

Immunohistochemistry characterization of cells within the lung capillaries of cirrhotic rats was performed on serial sections using a set of two monoclonal antibodies named ED1 and ED2, which label cells of the monocyte-macrophage lineage in rat (references M341 and M342, respectively; Dako; Glostrup, Denmark). ED1 monoclonal antibody labels a cytoplasmic antigen in both monocytes and free and tissue macrophages, while ED2 is directed toward membrane antigens found in tissue macrophages and Kupffer cells.30 Neutral formalin-fixed, paraffin-embedded tissue sections were processed by streptavidin-biotin-horseradish peroxidase method using diaminobenzidine as the final substrate.

Results

Animal Model of Cirrhosis

Neither clinical abnormality nor mortality was observed within the control groups (groups B and C). Cirrhotic rats (group A) developed persistent jaundice as early as the second day after surgery. A 20% mortality rate occurred within the first 7 days of surgery. Jaundice rapidly became extensive and included the ears and the limbs. Urine became darker, and the animals became somnolent and apathetic; at the end of the study, ascites was observed in many of the animals. No animals survived beyond 35 days.

Radiochemical Purity and Characterization of Radiopharmaceutical Batches

Radiochemical purity expressed as the ratio of radiolabeled peak areas of colloids to the total peak areas of the chromatogram was observed to be higher for batch B, with only 3% free 99mTc (Table 1). The analysis of batch A evidenced a nonhomogeneous distribution of particles (19% of the total amount of colloids corresponded to 41- to 207-nm particles and 81% to 227- to 447-nm particles), whereas the analysis of batch B showed particles ranging in size from 185 to 345 nm (Table 2). Biodistribution studies confirmed that the lower activity found within normal lung was obtained using batch B (Table 3). Considering batch radiochemical purity, size distribution of colloids, and minimal activity found in lung, batch B was considered to be the best suitable to achieve in vivo imaging of phagocytosis development within lung.

Tolerance of the Injection of Radiopharmaceutical

Neither mortality nor clinical side effects were noted in both control rats and cirrhotic rats following repeated IV injections of radiopharmaceutical.

Table 1—Radiochemical Purity of the Radioactive Colloid Batches

<table>
<thead>
<tr>
<th>Batch Formulation</th>
<th>Radiochemical Purity, %</th>
<th>150- to 400-nm</th>
<th>10- to 60-nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antimony sulfide</td>
<td>95</td>
<td>3</td>
<td>97</td>
</tr>
<tr>
<td>Batch A</td>
<td>93</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Batch B</td>
<td>97</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SD unless otherwise indicated.

Table 2—Particle Size Distribution of the Radioactive Colloid Batches

<table>
<thead>
<tr>
<th>Batch Formulation</th>
<th>Particle Size, nm</th>
<th>% of Particles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antimony sulfide</td>
<td>43 ± 15</td>
<td>100</td>
</tr>
<tr>
<td>Batch A</td>
<td>124 ± 83</td>
<td>19</td>
</tr>
<tr>
<td>Batch B</td>
<td>10 ± 1</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>265 ± 80</td>
<td>99.7</td>
</tr>
</tbody>
</table>

Table 3—In Vivo Biodistribution of the Radioactive Colloid Batches in Healthy Rats

<table>
<thead>
<tr>
<th>Batch Formulation</th>
<th>Biodistribution, % of Injected Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lung</td>
</tr>
<tr>
<td>Antimony sulfide</td>
<td>8.1</td>
</tr>
<tr>
<td>Batch A</td>
<td>7</td>
</tr>
<tr>
<td>Batch B</td>
<td>3</td>
</tr>
</tbody>
</table>
Quantitative Analysis of Scintigraphic Images and Blood Radioactivity Counting

Although presurgical values (Table 4) demonstrated exclusive liver uptake of particles for each of the 60 animals, a significant (p < 0.05) change in the biodistribution of radiopharmaceutical was observed for cirrhotic animals at day 35 as compared to control rats (Table 5). At this later stage of pathology, radiopharmaceutical uptake was significantly decreased within liver (30.7 ± 8.1% of total body activity) and considerably increased within lung (66.3 ± 5.3%). These differences were readily visualized on Figure 1, showing images of radiopharmaceutical biodistribution in a rat submitted to scintigraphy prior to surgery (Fig 1, top) and at end stage of pathology (Fig 1, bottom).

Figure 2 illustrates the progressive changes in radiopharmaceutical biodistribution as a function of time after biliary obstruction. Radiopharmaceutical uptake began to increase within lung from day 10 (7.9 ± 2.5%), liver uptake being 84.8 ± 4.5%. At day 20, the lung-uptake value was increased by a factor of 8, although liver uptake value was reduced by a factor of 1.5 (liver uptake 60.2 ± 11.5% and lung uptake 34.4 ± 8%). Day 27 showed a mean of 45% of radioactive particles distributed in both lung and liver. Then, lung uptake became predominant, leading at 35 days to a mean value of 66.3 ± 5.3%, while mean liver uptake was 30.7 ± 8.1%. Statistical analysis revealed that the increase in lung uptake for cirrhotic rats was significant (p < 0.05) from day 10 as compared to control rats. For liver, uptake decrease was slightly delayed and significant (p < 0.05) from day 20.

At day 35, values of blood activity were 5.2 ± 0.13% of the total radiopharmaceutical injected dose for control rats and 5.6 ± 0.12% for cirrhotic rats, showing a negligible amount of free radiopharmaceutical resident in the vascular space.

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**Table 4—Presurgical Values of Radiopharmaceutical Uptake Within Liver and Lung**

<table>
<thead>
<tr>
<th>Organs</th>
<th>Group A (n = 40)</th>
<th>Group B (n = 10)</th>
<th>Group C (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>91.7 ± 1.7</td>
<td>90 ± 2.9</td>
<td>91.2 ± 2.7</td>
</tr>
<tr>
<td>Lung</td>
<td>2.8 ± 0.1</td>
<td>3.1 ± 0.1</td>
<td>2.9 ± 0.3</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SD.

**Table 5—Values of Radiopharmaceutical Uptake Within Liver and Lung at Day 35**

<table>
<thead>
<tr>
<th>Organs</th>
<th>Group A (n = 40)</th>
<th>Group B (n = 10)</th>
<th>Group C (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>66.3 ± 5.3</td>
<td>91.2 ± 0.1</td>
<td>90.2 ± 0.1</td>
</tr>
<tr>
<td>Lung</td>
<td>30.7 ± 8.1</td>
<td>2.8 ± 0.1</td>
<td>2.4 ± 0.1</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SD.
Histology

Control Animals (Groups B and C): In the lung and liver of either sham operated-on or control rats, no lesions were detected (Fig 3, top).

Cirrhotic Rats (Group A): Histologic analysis of liver revealed features of cirrhosis with portal spaces considerably enlarged and dissection of hepatic lobules, necrosis, and fibrosis. Analysis of lung sections showed perivascular edema and hemorrhage. A large number of mononuclear cells within the capillaries exhibited features of macrophages with close adherence to the endothelium and abundant cytoplasm containing vacuoles (Fig 3, upper middle). From immunohistochemistry analysis, intravascular mononuclear cells within lung capillaries of cirrhotic rats stained for ED1 monoclonal (Fig 3, lower middle), but no specific staining of the adjacent section was observed with ED2 antibody (Fig 3, bottom).

Discussion

For many years, unexplained clinical observations of lung images resulting from IV injection of radiolabeled colloids commonly used for liver scintigraphy have been reported for patients with chronic cirrhosis, suggesting involvement of a RES function within the lung. Recent experimental data in cirrhotic rats focused the role of mononuclear phagocytic cells in this abnormal lung imaging pattern. These large mononuclear cells found in the pulmonary circulation of cirrhotic rats resembled in morphology and function the normal resident PIM population in sheep and pig.

From our results, the scintigraphic monitoring of each rat from initiation to the end stage of cirrhosis established in vivo for the first time the role of induced PIMs in lung imaging pattern; after a latent period, disease resulted in a dramatic impairment in RES function within liver associated with occurrence of the phagocytic function within lung. Quantitative analysis of the scintigraphic data and blood counting revealed that chronic biliary cirrhosis only resulted in biodistribution modification between liver and lung without any significant change in the total amount of radiopharmaceutical remaining in the vascular space.

Histologic examination revealed in animals with...
lung imaging pattern numerous mononuclear cells containing phagocytosis vacuoles and closely adherent to the endothelium. The cellular origin of these PIMs found in cirrhotic rats is as yet unknown, but authors have postulated that the migration of hepatic Kupffer cells could be at the origin of this phenomenon. Therefore, we tested this hypothesis using immunohistochemistry analysis of adjacent lung sections. These mononuclear cells showed selective labeling using the ED1 marker that is raised against rat monocytes but were not labeling using ED2 that is raised against tissue macrophages and Kupffer cells. No specific staining with either of these markers was observed in the sham operated-on or control animals. Thus, PIMs within lung capillaries of cirrhotic rats can now be considered as being derived from circulating monocytes rather than simply migrating Kupffer cells. Our results are in agreement with studies that showed that the majority of intravascular cells found within lung were activated monocytes exhibiting phagocytic properties.

Up to now, induction of phagocytic cells within the lung capillaries of cirrhotic rats had been correlated with an increased sensitivity of these animals toward endotoxins. From our results, progressive colonization of lung by PIMs was concomitant to increased mortality, suggesting a potential role of the ARDS. In species such as sheep and pig having natural PIMs, these cells have been demonstrated to release vasoactive and inflammatory mediators resulting in chronic inflammation and hemodynamic side effects.

Our experimental data in cirrhotic rats raise important questions whether such an analogous phenomenon could occur in patients with liver abnormality and/or RES dysfunction. Reported cases of lung uptake of colloidal radiopharmaceutical in the nuclear medicine literature tend to suggest that pulmonary phagocytosis can occur in human patients under pathologic circumstances. Thus, analysis of lung sections from cirrhotic patients who have identified pulmonary intravascular cells containing phagocytosis vacuoles resembling in morphology with PIMs described in species having them. If we hypothesize that PIMs could appear in the human lung under pathologic situations, the clinical consequence of pulmonary phagocytosis has to be considered: it includes both the risk of chronic lung inflammation and the potential risk of pulmonary hemodynamic side effects in response to IV injection of many drugs such as liposomes and contrast agents susceptible to be phagocytosed by PIMs. Although clinical studies are necessary to understand the pathologic implication of PIMs, scintigraphy of phagocytic function offers a suitable noninvasive strategy to establish the staging of pulmonary involvement in patients with liver failure.

**CONCLUSION**

For the first time (to our knowledge), in vivo scintigraphic monitoring of each rat from initiation to the end stage of pathology has demonstrated that biliary cirrhosis leads to the development of phagocytic cells within the lung. These cells are closely related to the monocyte-macrophage lineage. They are adherent to the pulmonary endothelium and are responsible for the lung uptake of radiopharmaceutical by phagocytosis. Similar scintigraphic monitoring of patients with liver failure should be performed to test whether PIMs are inducible in human, and, if so, this strategy could be of value to detect a population of patients susceptible to develop chronic and acute severe respiratory complications.

**REFERENCES**

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