Detection of Experimental Autoimmune Myocarditis in Rats by \(^{111}\) In Monoclonal Antibody Specific for Tenascin-C

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**Background**—Although the identification of inflammatory infiltrates in endomyocardial biopsy specimens is necessary for the definitive diagnosis of myocarditis, the biopsy test is invasive and is not sensitive. Therefore, a new diagnostic technique for the early and noninvasive evaluation of myocarditis has been awaited. Expression of tenascin-C (TNC), one of the oligometric extracellular glycoproteins, is induced in various pathological states, including inflammation, suggesting that TNC can be a molecular marker of myocarditis.

**Methods and Results**—An \(^{111}\) In anti-TNC monoclonal antibody Fab’ fragment was injected intravenously into rats with experimental autoimmune myocarditis (EAM), and the biodistribution of this radiotracer was measured. Rapid clearance of radioactivity from the blood was observed in both EAM and control rats (<1% at 6 hours after injection). Myocardial uptake of the tracer was much higher in EAM rats than in control rats (7.54-, 4.39-, and 3.51-fold at 6, 24, and 48 hours after injection, respectively). By autoradiography, high radioactivities were clearly observed in the regions indicative of inflammation in EAM rats. Single-photon emission CT imaging demonstrated the focal myocardial uptake of \(^{111}\) In anti-TNC Fab’ in vivo.

**Conclusions**—Radiolabeled anti-TNC Fab’ may be useful for the noninvasive diagnosis of myocarditis. (*Circulation*. 2002;106:1397-1402.)

***Key Words:*** myocarditis ■ antibodies ■ nuclear medicine
intravenously into EAM rats and control rats and measured its biodistribution. The localization of the radioactivity in the myocardium was also analyzed by ex vivo imaging (autoradiography) and in vivo imaging (single-photon emission CT [SPECT]).

Methods

Induction of EAM
Forty female 7-week-old Lewis rats purchased from Charles River Japan Inc (Atsugi, Kanagawa, Japan) were used under the protocol approved by the Special Committee on Animal Welfare of the Inohana Campus of Chiba University. EAM was induced in rats as previously described.23 In brief, rats were immunized twice at a 7-day interval with 10 mg/mL porcine cardiac myosin (Sigma Chemical Co) with Freund’s complete adjuvant alone (control rats). All experiments were performed at 17 days after the first immunization.

Antibody and Western Blotting
A mouse monoclonal antibody against TNC, clone 4F10TT, was raised by immunization of a TNC-null mouse19 with purified human TNC as described previously.21 Isolated splenic cells from a TNC-null mouse immunized with TNC were fused with SP2/0 myeloma cells. Immunoglobulin was purified from the culture supernatant of hybridoma cell clone 4F10TT cultured in serum-free media. The Fab’ fragments of the antibody were prepared by reducing the disulfide bonds of the F(ab)’ fragments with mercaptoethanol after pepsin digestion of the intact antibody.

As a nonspecific control antibody, an isotype-matched monoclonal antibody against the V protein of paramyxovirus type II (clone 53-1, isotype IgG1; kindly provided by the Department of Microbiology, Mie University School of Medicine) was also digested to the Fab’ fragment as described above.

Immunoblotting was performed as previously described.20 In brief, hearts were removed from rats on day 21, and the myocardium was homogenized in 4 mL buffer A (20 mmol/L Tris-HCl, 0.15 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L diithiothreitol, pH 7.4). Samples of protein (20 μg/lane) were subjected to SDS-PAGE with 2% to 15% gradient polyacrylamide gel and electrophoretically transferred onto Immobilon membranes (Millipore). The transferred proteins were immunostained with 4F10TT (1 μg/mL) by using the indirect immunoperoxidase method.

Immunohistochemical Staining
Immunostaining of tissue sections was performed as described previously.21 In brief, the sections were first incubated with primary antibodies 4F10TT (1 μg/mL) and then with peroxidase-conjugated anti-mouse IgG (1:500, MBL, Nagoya, Japan). After the sections were washed, diaminobenzidine/H2O2 solution was used to demonstrate antibody binding. The slides were lightly counterstained with hematoxylin for microscopic examination.

Radiolabeling With 111In
The purified conjugates were labeled with 111In, as previously reported.23,24 In antibody fragments were purified by a centrifuged column procedure using Sephadex G-50 equilibrated with 0.1 mol/L PBS (pH 6.0). Radiochemical purities of the 111In antibody fragments were determined by cellulose acetate electrophoresis and size-exclusion high-performance liquid chromatography.

Biodistribution Study
Ten EAM rats and 10 control rats were injected with 100 μg (111 KBq) of 111In anti-TNC Fab’ in 300 μL saline solution intravenously through the tail vein. Groups of 3 or 4 animals were euthanized at 6, 24, and 48 hours after the tracer injection. Hearts, other organs (lung, liver, kidney, spleen, cerebrum, foot pad, and hindlimb muscle), and blood were weighed and then counted for radioactivity with the blood samples. Pleural effusion was also sampled in EAM rats. The percentage of the injected dose of the radioactivity per gram tissue weight was calculated. To evaluate the dose-dependent changes in EAM-targeting properties of the radiotracer, groups of 3 rats each were administered different amounts of 111In anti-TNC Fab’ (10, 50, or 100 μg per rat) and euthanized at 6 hours after injection, and the uptake of the radiotracer in the heart and other organs was estimated.

The specific uptake of anti-TNC antibody in EAM rats was also estimated at 6 hours after injection, the radioactivity distribution of the same amounts (3 μCi, 50 μg) of 111In anti-TNC Fab’ (n=3) or 111In nonspecific Fab’ (n=5).

Histopathologic and Quantitative Autoradiographic Study
Two groups of EAM rats were administered similar amounts (740 KBq, 50 μg) of either 111In anti-TNC Fab’ or 111In nonspecific Fab’. A similar amount of 111In anti-TNC Fab’ was also injected into control rats. At 6 hours after injection, the hearts were excised and embedded in Tissue-Tek OCT compound (Miles). Sections of 5-μm thickness were mounted on a slide and stained with hematoxylin and eosin (H-E) for pathological analysis. Sections of 20-μm thickness were exposed to autoradiography. The imaging plate was exposed for 24 hours and studied by image analyzer (BAS-1800 system, Fuji Film Co Ltd). Autoradiographic intensities of each myocardial tissue were presented as (PSL–BG)/A, where PSL is photostimulated luminescence, BG is PSL of the background, and A is area (mm²).

In Vivo Imaging
111In anti-TNC Fab’ in vivo imaging was performed in an EAM rat by use of SPECT. The SPECT system consisted of a 3-headed γ-camera (Toshiba GCA 9300A) equipped with 1.0-mm pinhole collimators.111In anti-TNC Fab’ (2.2 MBq) was injected intravenously, and after 5.5 hours, 37 MBq of 99mTc sestamibi (MIBI) (DRL, Tokyo, Japan) was injected. Thirty minutes later, a rat was anesthetized by an intraperitoneal injection of pentobarbital (30 μg/g), and 60-minute data acquisition was performed at 120 seconds per view with stepwise rotation for each 4° over 120° and with multiple peak acquisition (15% windows for the 99mTc and both 111In peaks). The matrix was 128×128 for data acquisition and for the image display with 0.6-mm pixel size.

Results

Expression of TNC in EAM Hearts
Western blot analysis of the myocardium showed that in the EAM rat, 2 strong bands were observed at ~270 and 230 kDa (Figure 1; arrowheads, lane 2), whereas no band was observed in the normal rat myocardium (lane 1). In human gliaoma TNC, 3 bands were detected at ~300, 240, and 210 kDa (arrows, lane 3). At 21 to 28 days after the first injection of the antigen, severe inflammatory cell infiltration and myocyte necrosis were recognized throughout the myocardium (Figure 2b). Strong immunostaining for TNC was observed in the inter-
stitutum. On the other hand, no immunostaining was detected in the normal myocardium (Figure 2a).

In Vivo Biodistribution of $^{111}$In Anti-TNC Fab’
The numbers of EMCS-Bz-EDTA groups attached per antibody molecule were 1.4 and 1.6 for anti-TNC antibody and control antibody, respectively. Fewer than 0.05 free thiol groups were found per molecule of each antibody fragment after the addition of iodoacetamide. After purification by centrifuged column procedure, $^{111}$In anti-TNC Fab’ and $^{111}$In nonspecific Fab’ were obtained with radiochemical purities of 96.9% and 97.6% for anti-TNC antibody and the nonspecific antibody, respectively.

The tissue uptake of $^{111}$In anti-TNC Fab’ is summarized in Table 1 as a percentage of the injected dose per gram tissue. Rapid clearance of radioactivity from the circulation was observed with $^{111}$In anti-TNC Fab’ in both EAM and control rats (<1% at 6 hours after injection). The kidney uptake was the highest at all time points, with the spleen and liver being next and then the myocardium. However, myocardial uptake was significantly lower in control rats compared with EAM rats. Radioactivity was much higher in EAM rat hearts than in control rat hearts; the increases were 7.54-,, 4.39-,, and 3.51-fold at 6, 24, and 48 hours, respectively. Heart-to-blood ratios of radioactivity were much higher in EAM rats than in control rats and increased gradually after injection (Table 1). Comparative myocardial uptakes with adjoining organs, such

Table 1. Biodistribution of $^{111}$In-Labeled Anti-TNC Fab’ in EAM and Control Rats

<table>
<thead>
<tr>
<th>Tissue</th>
<th>6 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAM rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>0.45±0.08</td>
<td>0.12±0.02</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>PE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>2.39±0.17</td>
<td>1.06±0.13</td>
<td>0.59±0.20</td>
</tr>
<tr>
<td>Lung</td>
<td>0.76±0.08</td>
<td>0.48±0.06</td>
<td>0.48±0.17</td>
</tr>
<tr>
<td>Liver</td>
<td>3.05±0.26</td>
<td>1.70±0.33</td>
<td>1.20±0.27</td>
</tr>
<tr>
<td>Kidney</td>
<td>20.78±1.38</td>
<td>18.69±3.63</td>
<td>19.12±4.90</td>
</tr>
<tr>
<td>Spleen</td>
<td>6.00±0.46</td>
<td>5.94±1.23</td>
<td>4.49±1.13</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>0.01±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>Foot pad</td>
<td>0.49±0.04</td>
<td>0.58±0.27</td>
<td>0.52±0.18</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.15±0.17</td>
<td>0.09±0.04</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>Heart/blood</td>
<td>5.35±0.75</td>
<td>8.81±1.62</td>
<td>12.36±5.40</td>
</tr>
<tr>
<td>Heart/lung</td>
<td>3.17±0.38</td>
<td>2.25±0.34</td>
<td>1.44±0.84</td>
</tr>
<tr>
<td>Heart/liver</td>
<td>0.79±0.07</td>
<td>0.65±0.16</td>
<td>0.52±0.22</td>
</tr>
<tr>
<td>Control rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>0.88±0.08</td>
<td>0.21±0.02</td>
<td>0.08±0.00</td>
</tr>
<tr>
<td>Heart</td>
<td>0.32±0.03</td>
<td>0.24±0.02</td>
<td>0.17±0.01</td>
</tr>
<tr>
<td>Lung</td>
<td>0.50±0.06</td>
<td>0.33±0.03</td>
<td>0.24±0.00</td>
</tr>
<tr>
<td>Liver</td>
<td>4.51±0.55</td>
<td>2.41±0.34</td>
<td>1.67±0.10</td>
</tr>
<tr>
<td>Kidney</td>
<td>13.34±2.36</td>
<td>14.75±3.34</td>
<td>16.78±2.89</td>
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<tr>
<td>Spleen</td>
<td>7.30±0.57</td>
<td>9.04±0.76</td>
<td>7.84±0.58</td>
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<tr>
<td>Cerebrum</td>
<td>0.02±0.00</td>
<td>0.01±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>Foot pad</td>
<td>0.47±0.07</td>
<td>1.28±0.58</td>
<td>0.50±0.06</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.05±0.04</td>
<td>0.03±0.01</td>
<td>0.05±0.04</td>
</tr>
<tr>
<td>Heart/blood</td>
<td>0.36±0.00</td>
<td>1.16±0.18</td>
<td>2.19±0.12</td>
</tr>
<tr>
<td>Heart/lung</td>
<td>0.64±0.03</td>
<td>0.74±0.05</td>
<td>0.70±0.05</td>
</tr>
<tr>
<td>Heart/liver</td>
<td>0.07±0.00</td>
<td>0.10±0.01</td>
<td>0.10±0.01</td>
</tr>
</tbody>
</table>

Values at each time point represent mean±SD of percentage of injected dose per gram tissue weight. Each EAM or control rat received 0.3 mL $^{111}$In anti-TNC Fab’ in saline (3 μCi IV).

PE indicates pulmonary effusion (nonexistent in control rats); muscle, hindlimb skeletal muscle.

Figure 2. Immunostaining for TNC of sections from normal rat myocardial tissue (a) and from rat myocardial tissue at day 21 after first injection of myosin (b). Bar=50 μm (a).
TABLE 2. Comparison of Biodistribution Between $^{111}$In Anti-TNC Fab’ and Nonspecific Fab’ in EAM Rats at 6 h After Injection

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Anti-TNC Fab’</th>
<th>Nonspecific Fab’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.74±0.06</td>
<td>0.09±0.03</td>
</tr>
<tr>
<td>PE</td>
<td>0.56±0.03</td>
<td>0.42±0.09</td>
</tr>
<tr>
<td>Heart</td>
<td>2.60±0.32</td>
<td>0.32±0.06</td>
</tr>
<tr>
<td>Lung</td>
<td>0.64±0.03</td>
<td>0.22±0.06</td>
</tr>
<tr>
<td>Liver</td>
<td>6.04±0.45</td>
<td>0.50±0.10</td>
</tr>
<tr>
<td>Kidney</td>
<td>15.78±1.67</td>
<td>53.66±1.26</td>
</tr>
<tr>
<td>Spleen</td>
<td>8.53±0.42</td>
<td>1.00±0.12</td>
</tr>
<tr>
<td>Foot pad</td>
<td>0.71±0.09</td>
<td>0.26±0.09</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.06±0.05</td>
<td>0.16±0.08</td>
</tr>
</tbody>
</table>

Values at each time point represent mean±SD of percentage of injected dose per gram tissue weight. Each EAM rat received 0.3 mL $^{111}$In anti-TNC Fab’ or nonspecific Fab’ in saline (3 μCi IV).

as the lung and liver, are also shown in Table 1. The radiotracer reached its highest heart-to-liver and heart-to-lung ratios of radioactivity at 6 hours after injection.

The dose-dependent changes in biodistribution of $^{111}$In anti-TNC Fab’ in EAM rats showed that the tracer uptake was higher with 10 μg radiolabeled antibodies in the liver and spleen than with 50 or 100 μg radiolabeled antibodies. In the heart, the tracer uptake was lower with 100 μg antibodies than with 10 or 50 μg antibodies. Thus, the antibody dose of 50 μg per rat was used in further experiments.

The comparison of radioactivity distribution between anti-TNC antibody and nonspecific antibody in EAM rats is summarized in Table 2. In the heart, much higher radioactivity was observed with an injection of $^{111}$In anti-TNC Fab’ compared with an injection of $^{111}$In nonspecific Fab’ (2.60±0.32 for $^{111}$In anti-TNC Fab’ versus 0.32±0.06 for $^{111}$In nonspecific Fab’). Radioactivity of $^{111}$In anti-TNC Fab’, compared with $^{111}$In-nonspecific Fab’, was lower in the kidney and higher in the blood, liver, and spleen.

**Histopathologic and Quantitative Autoradiographic Study**

By autoradiography, high radioactivities were observed in the region indicative of inflammation in EAM rats administered $^{111}$In anti-TNC Fab’ (Figure 3, row 1). No accumulation of radioactivity was observed in EAM rats with $^{111}$In nonspecific Fab’ (row 2) or in normal rats with $^{111}$In anti-TNC Fab’ (row 3).

The ratios of autoradiographic intensities of each myocardial tissue in each rat group are summarized in Table 3. Because the original autoradiographic images were very small, regions of interest were placed on the autoradiograms of the whole myocardium in this analysis. High accumulation of $^{111}$In anti-TNC Fab’ in EAM rat hearts was clearly shown.

**In Vivo Imaging**

Finally, we examined whether $^{111}$In anti-TNC Fab’ imaging could be used to detect in vivo myocardial injury. SPECT imaging (Figure 4a) and autoradiography (Figure 4d) clearly showed the focal uptake of $^{111}$In anti-TNC Fab’ (Figure 4a). $^{99m}$Tc-MIBI myocardial perfusion imaging (Figure 4b) demonstrated the image complementary to $^{111}$In anti-TNC Fab’ (Figure 4c).

**Discussion**

Our results demonstrate that the myocardial uptake of $^{111}$In anti-TNC Fab’ was significantly higher in EAM rats than in control rats. By autoradiography, high radioactivities were clearly observed in the regions indicative of inflammation in EAM rats. SPECT imaging demonstrated the suitability of this tracer for in vivo imaging.

We have recently reported that TNC was expressed at the initial stage of myocarditis in an autoimmune mouse model before necrosis or inflammatory cell infiltration was histologically apparent. This result reveals the potential of early

**TABLE 3. Autoradiographic Intensity Averages**

<table>
<thead>
<tr>
<th>Average (PSL−BG)/A Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>(EAM–Anti-TNC)/(control–anti-TNC)</td>
</tr>
<tr>
<td>(EAM–anti-TNC)/(EAM–NS)</td>
</tr>
<tr>
<td>(EAM–NS)/(control–anti-TNC)</td>
</tr>
</tbody>
</table>

PSL indicates photostimulated luminescence; BG, PSL of background; A, area (mm²); anti-TNC, anti-TNC antibody; and NS, nonspecific antibody.

Words in parentheses in first column show combinations of rats and monoclonal antibodies used in experiment. For example, (EAM–anti-TNC) indicates average autoradiographic intensities in EAM rat hearts injected with radiolabeled anti-TNC antibody. Regions of interest were placed on autoradiogram of whole myocardium.
The focal uptake of 111In anti-TNC Fab′ is highly specific to the inflammatory region of myocarditis. This was strongly supported by SPECT imaging, in which focal uptake of 111In anti-TNC Fab′ was clearly demonstrated at sites complementary to 99mTc-MIBI uptake (Figure 4). This finding also suggests that anti-TNC antibody fragments would be useful for clinical imaging, although further manipulation of the radioactivity levels in the blood, kidney, and liver is necessary.

Although inflammation and the expression of TNC in EAM rats peak at 21 days after the first immunization,17 all experiments were performed at 17 days because the hemodynamic abnormalities are too severe at 21 days to evaluate the kinetics of the tracer. In EAM rats, there is a characteristic initial focal inflammatory response in the myocardium, followed by massive myocardial damage at 21 days. In addition, the extent of TNC expression at 17 days is somewhat variable, as shown in Figure 3, a and b, and in Figure 4.

In conclusion, we investigated the inherent biodistribution of anti-TNC antibody Fab′ fragments in the EAM rat model. The present study indicated a high ability of the antibody fragment to localize sites of inflammation in the heart by ex vivo and in vivo imaging. Although further studies are required, radiolabeled anti-TNC fragments may be an attractive radiopharmaceutical for the noninvasive diagnosis of myocarditis.

**Figure 4.** Transverse dual-isotope tomographic images of 111In anti-TNC Fab′ and 99mTc-MIBI in EAM rat. a. Arrow shows focal uptake of 111In anti-TNC Fab′. b. 99mTc-MIBI myocardial perfusion image is shown. c. Superimposed image demonstrated that focal uptake of 111In anti-TNC Fab′ was detected on interventricular septum (S). d. Autoradiography. A indicates anterior; L, lateral; and P, posterior.

In the present study, the anti-TNC antibody fragment was labeled with 111In by using EMCS-Bz-EDTA, so that the radioactivity provides inherent in vivo behaviors of the antibody fragment.22,23 Western blot analysis confirmed that the anti-TNC monoclonal antibody, 4F10TT, bound to 2 isoforms of rat TNC (Figure 1). Specific accumulation of 4F10TT in the inflammatory myocardium was confirmed by significantly lower accumulation of the nonspecific antibody in the hearts of EAM rats (Table 2). The regions of high accumulation of 111In anti-TNC Fab′ in ex vivo imaging (autoradiography) coincided well with the regions of inflammatory cell infiltration (Figure 3), indicating that anti-TNC Fab′ is highly specific to the inflammatory region of myocarditis.

References
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