

Imaging of lesions in a murine rheumatoid arthritis model with a humanized anti-interleukin-6 receptor antibody

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Rheumatoid arthritis (RA) has been attributed to the abnormal production of cytokine interleukin-6 (IL-6), which has a variety of physiological activities. *In vivo* IL-6-receptor imaging provides useful suggestions regarding the mechanism of anti-IL-6-receptor antibody action and indicates a basic therapeutic strategy for treating RA. Therefore, this study was designed to establish a method for radiolabeling anti-IL-6-receptor antibodies and to investigate the feasibility of using radiolabeled anti-IL-6-receptor antibodies in the scintigraphic imaging of lesions in an animal RA model. Anti-IL-6-receptor antibodies were conjugated with a bifunctional chelating agent, hydrazinonicotinamide (HYNIC), and radiolabeled with technetium-99m (^{99m}Tc) using the ligand exchange reaction of ^{99m}Tc -tricine complex. The binding affinity was estimated using the U266 cell line. Whole body scintigraphy, biodistribution and autoradiography were undertaken in mice containing synovial cells that had been transplanted from an RA patient. Our findings showed that the antibodies accumulated in the implanted tissue. When radiolabeled anti-IL-6-receptor antibodies are used in scintigraphic imaging, the distribution of the IL-6-receptors is associated with the inflammatory cell infiltration that is seen in the early stage of RA. Accordingly, imaging with humanized anti-IL-6-receptor antibodies appears to be useful for detecting early pathophysiological conditions and assessing the efficacy of antibody treatment as well as the prognosis of patients with RA.

Key words: rheumatoid arthritis, interleukin-6, HYNIC, technetium-99m, scintigraphic imaging

INTRODUCTION

RHEUMATOID ARTHRITIS (RA) is a systemic chronic inflammatory disease characterized by synovial proliferation in multiple joints and progressive bone and joint changes or destruction with time. Many cytokines are involved in the pathogenesis of RA, and interleukin-1, interleukin-6 (IL-6), and tumor necrosis factor- α play important roles in the

inflammatory process.¹ IL-6 has been shown to stimulate plasmacytes to secrete immunoglobulin,² activate and promote the proliferation of T lymphocytes,³ increase the number of neutrophils and platelets under conditions of anemia,⁴ induce acute-phase proteins such as C-reactive protein (CRP), fibrinogen, haptoglobin, and serum amyloid A,⁵ regulate the proliferation and differentiation of osteoclasts, and induce bone resorption.⁶ RA is associated not only with joint manifestations but also with other clinical manifestations such as fever, malaise, anemia, excessive platelet counts, elevated CRP, elevated sedimentation rate, hypoalbuminemia, and hypergammaglobulinemia. In addition, serum and synovial fluid IL-6 levels are increased in patients with RA.^{7,8} All of these findings suggest that such joint and other clinical manifestations of RA may be attributable to the abnormal production of IL-6.

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Therefore, assuming that RA could be successfully treated if IL-6 signals could be controlled, an attempt is being made to treat RA by inhibiting the action of IL-6 with humanized anti-interleukin-6 receptor antibody.^{9,10} Although the pathogenesis of RA has frequently been investigated by monitoring the IL-6 concentration in blood and tissues, the mechanism responsible for the distribution of IL-6 receptors remains uncertain. It has been shown that the expression of IL-6 receptors occurs frequently in RA lesions, and that RA patients are likely to improve if they are treated with anti-IL-6 receptor antibodies,^{11,12} suggesting that anti-IL-6 receptor antibodies specifically recognize the IL-6 receptors in RA lesions. If scintigraphic imaging with radiolabeled anti-IL-6 receptor antibodies becomes feasible, the biodistribution of the radiolabeled anti-IL-6 receptor antibodies would be helpful in detecting early RA lesions and assessing the therapeutic efficacy of treatment with anti-IL-6 receptor antibodies. This study was thus designed to establish a method for radiolabeling anti-IL-6 receptor antibodies and to investigate the feasibility of using radiolabeled anti-IL-6 receptor antibodies in the scintigraphic imaging of lesions in an animal RA model.

MATERIALS AND METHODS

Preparation of HYNIC-conjugated antibody

We used humanized anti-IL-6 receptor antibody (rhPM-1), which was made from the chimeric anti-IL-6 receptor antibody of mouse.⁹ Technetium-99m (^{99m}Tc) was selected as the radionuclide to be used for labeling after taking into account its application in single photon emission computed tomography (SPECT) and the kits that are commercially available for radiolabeled antibodies. The biofunctional reagent, hydrazinonicotinamide (HYNIC),¹³ for labeling was coupled to the antibody because it produces stable binding to ^{99m}Tc. First, succinimidyl-6-hydrazinopyridine-3-carboxylate hydrochloride was synthesized as described by Abrams et al.¹³ Succinimidyl-6-hydrazinopyridine-3-carboxylate hydrochloride was dissolved in DMSO, and the concentration was adjusted to 1.55 mg/ml. 10 μ l of this solution was added to 100 μ l of the antibody solution (2 mg/ml in PBS, pH = 7.4). After incubation at room temperature for 2 hours, the mixture was purified using gel filtration in a centrifuged column (Sephadex-G50). The protein concentration of the product was determined using a spectrometer at an absorption of 280 nm.

Radiolabeling of HYNIC conjugated antibody

^{99m}Tc-labeling was performed through a ligand exchange. For the labeling of the HYNIC-conjugated antibody with ^{99m}Tc, tricine was used as an intermediary ligand, as described by Lasen et al.¹⁴ A volume of 1 ml of ^{99m}Tc-pertechnetate was added to a vial containing sterile lyophilized stannous sulphate and tricine. After incubation at

room temperature for 15 minutes, 100 μ l was removed from the vial and added to a tube containing 100 μ l of HYNIC-conjugated antibody solution. After incubation at room temperature for 30 minutes, the mixture was purified using gel filtration in a centrifuged column (Sephadex-G50). The radiochemical purity of the labeled antibodies was measured by cellulose-acetate electrophoresis.

Binding assay of radiolabeled antibody to U266 cells

The physiological activity of the labeled antibodies was measured by binding assay using U266 cells, which are known to induce the expression of IL-6 receptors. U266 cells for assay were washed twice with 0.05 M phosphate buffer and incubated in a binding medium (RPMI Medium 1640 with 10% FCS) for at least 10 min at 37°C. The radiolabeled antibody was diluted with 0.1 M PBS (pH 7.4) to adjust the protein concentration to 6 μ g/ml, and 6 concentrations were made by serial dilutions. A total of 10⁶ cells were mixed with these radiolabeled antibody solutions in a final volume of 200 μ l of binding medium and incubated on ice for 1 hour, with occasional agitation every 10–15 min. At the end of the incubation period, the reaction mixture was centrifuged at 3000 rpm for 15 sec. The medium was exchanged for the new one, and the cell pellet was suspended again. This washing operation was repeated three times. Finally, the binding medium was removed, and the cell-associated radioactivity was measured using a gamma counter.

Serum stability of radiolabeled antibodies

The stability of the labeled antibodies in blood was measured by incubation in human plasma. Radiolabeled antibodies were diluted with 0.1 M PBS (pH 7.4) to adjust the protein concentration to 5 μ g/ml, and 20 μ l of this solution was added to a mixture of 115 μ l of freshly prepared human serum. After incubation for 0.5, 1, 3, 18 and 24 hours at 37°C, samples were removed from the reaction mixture. The percentage of radioactivity bound to the antibodies was determined using TLC developed with acetone. Radiolabeled antibodies and free ^{99m}Tc exhibited respective *R_f* values of 0–0.05 and 0.9–0.95 in this TLC system.

Preparation of RA model mice

An RA model was prepared by subcutaneously implanting synovial cells from an RA patient into dorsal sites of severe combined immunodeficient (SCID) mice (18–20 g).¹⁵

Analysis of human IL-6 in the blood of RA model mice

Blood sampling from RA model mice was performed once every week after the implantation of human synovial cells. The quantity of human IL-6 was measured by chemiluminescence enzyme immuno assay (CLEIA).

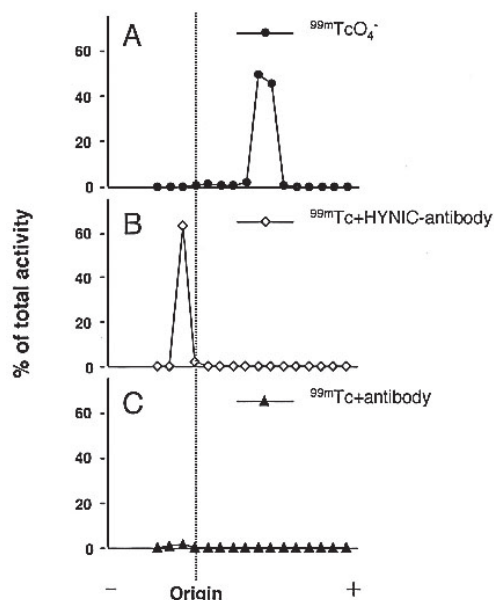


Fig. 1 Cellulose acetate electrophoresis of $^{99m}\text{TcO}_4^-$ (A), ^{99m}Tc -labeled HYNIC-coupled humanized anti-interleukin-6 receptor antibody (^{99m}Tc -HYNIC-antibody) (B), and the reaction solution of ^{99m}Tc -tricine₂ and unmodified antibody (C). Cellulose acetate electrophoresis was performed in a veronal buffer (I = 0.06, pH 8.6) with an electrostatic field of 0.8 mA/cm for 45 min. After gel filtration by centrifuged column, a radiochemical purity of 93.7% was obtained for the ^{99m}Tc -HYNIC-antibody. The reaction of ^{99m}Tc -tricine₂ with the unmodified antibody resulted in an absence of radioactivity within the protein fraction.

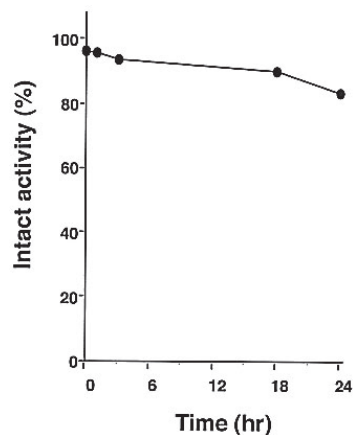


Fig. 3 Stability of ^{99m}Tc -HYNIC-antibody in human serum at 37°C as determined by cellulose acetate electrophoresis. A weak desorption of radioactivity from the antibody can be observed. However, more than 85.7% of the radioactivity was retained within the protein fraction 24 hours after incubation.

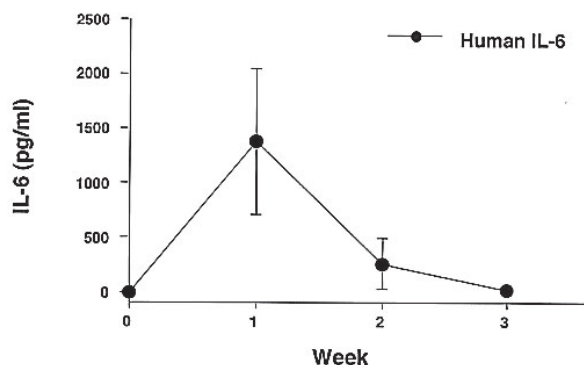


Fig. 4 Level of human IL-6 in blood of RA model mice (n = 4).

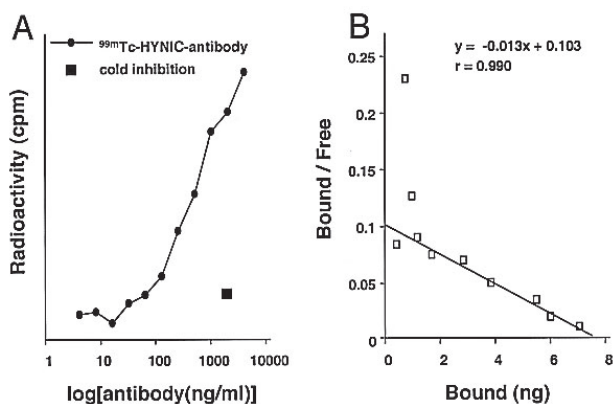


Fig. 2 Binding of ^{99m}Tc -HYNIC-antibody to U266 cells as a function of antibody concentration. A marked decrease was observed in ^{99m}Tc -HYNIC-antibody binding when 1000-fold (30 μg) of cold antibody was added (A). The Scatchard analysis shows a good correlation: $K_d = 2.56 \times 10^{-9}$ M, and $B_{\text{max}} = 7.92$ ng (B).

Biodistribution of radiolabeled antibodies in mice

Radiolabeled antibodies were diluted with 0.1 M PBS (pH 7.4) and cold antibodies to adjust the protein concentration to 200 $\mu\text{g}/\text{ml}$. 100 μl of this solution was injected in RA model SCID mice 2 weeks after the implantation of

synovial cells from an RA patient. SPECT scan was performed with a pinhole collimator to acquire the high resolution image. 6 hours after the injection of the radio-labeled antibody, SPECT images were obtained while the mice were under anesthesia. Similarly prepared ^{99m}Tc -labeled non-specific HYNIC-coupled human IgG antibodies were used as a control. The mice were then sacrificed using an intraperitoneal injection of phenobarbital. Tissues of interest were immediately removed and weighed, and the radioactivity was determined using a well counter. For histological and autoradiographic examination on the synovial tissue sections, the tissues were fixed in paraformaldehyde, dehydrated, and embedded in paraffin. Sections (5 μm) were placed in contact with autoradiography imaging plates and left for 3 days, and then stained with hematoxylin-eosin. An autoradiographic image was obtained and analyzed with a bio-imaging analyzer (BAS-5000, Fujifilm).

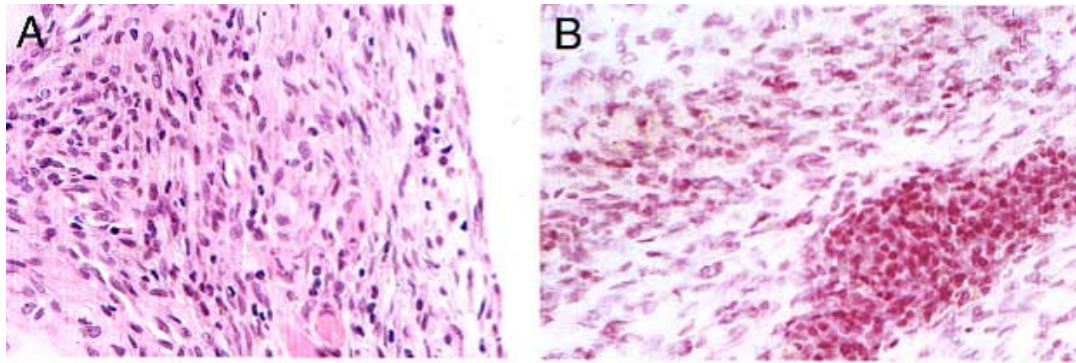


Fig. 5 Histological appearance of human synovial tissue before implantation in SCID mice (A), and implanted tissue in SCID mice engrafted with human rheumatoid tissue, 2 weeks after implantation (B).

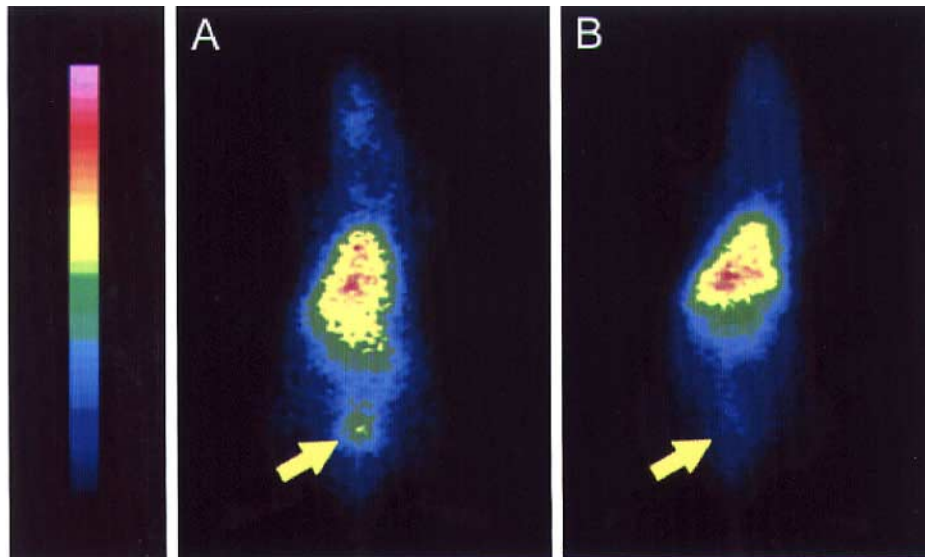


Fig. 6 Images of the same mouse 6 hours after the injection of ^{99m}Tc -HYNIC-antibody (A) and non-specific human IgG antibody (B). The yellow arrowhead points to the implanted tissue area.

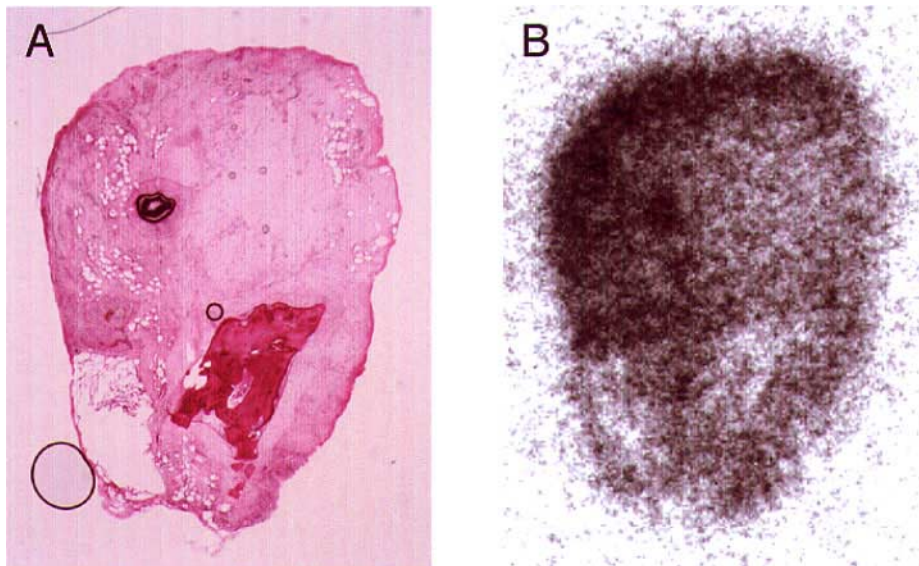


Fig. 7 Hematoxylin-eosin staining pattern (A) and autoradiogram (B) of the same tissue section. A good correlation can be seen between the density of the synovial cells and the tracer uptake, suggesting that tracer uptake depends on the IL-6 receptor recognition of the anti-IL-6 receptor antibody.

RESULTS

When anti-IL-6 receptor antibodies coupled to HYNIC were quantitatively determined in the hydrazide group, an average of 1.11 HYNIC molecules was found to be coupled to a single antibody molecule. Cellulose acetate electrophoretic analysis revealed the radiochemical yield to be 63.0% and the radiochemical purity to be 93.7% after labeling with ^{99m}Tc (Fig. 1A–B). No evidence of radioactivity was found in the antibody fractions where the reaction of untreated-antibodies to the ^{99m}Tc -tricine complex would have occurred (Fig. 1C).

The binding assay of the radiolabeled antibodies to U266 cells confirmed that the degree of binding increased in association with a rising concentration of the labeled antibodies. The degree of binding decreased in the presence of about 1000-fold cold antibodies. The graph had not reached a plateau state at the end of the plot. But, because this was the maximum concentration that we were able to obtain, we could not observe the data of higher concentrations from this assay (Fig. 2A). According to a Scatchard analysis, the results obtained from the binding assay were expressed as follows: $K_d = 2.56 \times 10^{-9}$ M and $B_{\text{max}} = 7.92$ ng. U266 cells were found to express IL-6 receptors on 11200 sites/cell (Fig. 2B). These results are very similar as compared with the values in a past article.¹⁶ Therefore, we thought that this radiolabeled antibody maintained a sufficient ability of IL-6 receptor recognition.

About the serum stability of radiolabeled antibodies, 85.7% of the radioactivity remained intact in the antibodies 24 hours after incubation in human serum at 37°C (Fig. 3).

Figure 4 shows the change in human IL-6 levels in the blood of RA model mice ($n = 4$). Human IL-6, which does not naturally exist in mouse blood, is obviously being secreted. The highest secretion value was measured at 1 week after implantation. In addition, a hematoxylin-eosin stained image of the implanted tissue had almost the same histological appearance as a specimen of human synovial tissue that is made before implantation. The proliferation of synovial villi, stratification of the synovial layers and infiltration by inflammatory cells can be observed in implanted tissue, similarly to human synovial tissue (Fig. 5).

Regarding the biodistribution study, large amounts of radiolabeled anti-IL-6 receptor antibodies tended to be retained in the blood ($20.3 \pm 4.51\%$ ID/g tissue) 6 hours after their injection into the caudal vein (Table 1). Blood clearance of the antibody is known to be slow, and this result was anticipated. However, the tissue/muscle ratio of the radioactivity accumulation showed 2.4, and a visible accumulation of radioactivity in the implanted tissue was observed on SPECT images obtained 6 hours after the injection of the radiolabeled anti-IL-6 receptor antibodies (Fig. 6). Furthermore, autoradiography of the

Table 1 Biodistribution study in RA model mice 6 hours after the injection of ^{99m}Tc -HYNIC-antibody

	% ID/g tissue (n = 3)	Muscle ratio
Blood	20.3 ± 4.51	25.5 ± 6.71
Liver	6.85 ± 1.72	8.45 ± 1.75
Kidneys	5.90 ± 2.84	6.86 ± 1.85
Spleen	16.4 ± 3.37	20.7 ± 6.05
Lung	10.9 ± 3.43	13.6 ± 4.01
Heart	5.28 ± 1.46	6.59 ± 1.80
Intestine	2.47 ± 0.64	3.05 ± 0.70
Stomach	1.57 ± 0.62	1.90 ± 0.46
Tumor	2.11 ± 1.58	2.40 ± 1.40
Muscle	0.85 ± 0.32	—

tissue section showed that tracer uptake depended on the density of the synovial cells (Fig. 7). On the other hand, radioactivity was not seen in the control RA model, which was injected with ^{99m}Tc -labeled non-specific HYNIC coupled to human IgG antibodies.

DISCUSSION

The purposes of our study were to develop radiolabeled anti-IL-6 receptor antibodies and to investigate the feasibility of their use in scintigraphic imaging. Cellulose acetate electrophoretic analysis revealed that HYNIC specifically coupled with the ^{99m}Tc -tricine complex. The binding assay suggested that the labeled antibodies specifically recognized the IL-6 receptors. The stability of ^{99m}Tc labeled compounds in blood is very important because the desorption of free ^{99m}Tc would cause the accumulation of the radioactivity in an unspecific region, such as the stomach or intestine. Regarding our ^{99m}Tc labeled compound, the binding of ^{99m}Tc and the HYNIC-conjugated antibody was very stable in human serum. These results suggested that stable antibody labeling conditions were established, and that the physiological activity of anti-IL-6 antibodies was maintained even after labeling with ^{99m}Tc . Then, we regarded that this compound could be used for scintigraphic imaging.

On the other hand, the appropriateness of the RA model was evaluated by physiological analysis. Human IL-6 secretion in mouse blood and the histological appearance of the implanted tissue suggested that the implanted tissue was alive and retained its characteristics while in the mouse body. So, it is conceivable that the transplantation succeeded.

Based on the biodistribution of the radiolabeled anti-IL-6 receptor antibodies and the SPECT and autoradiographic findings, the antibodies appeared to accumulate in the implanted tissue. This accumulation was judged to be specific because human IgG antibodies having the same molecular weights as the anti-IL-6 receptor antibodies did not accumulate in the implanted tissues. These findings suggest that the accumulation of radiolabeled anti-IL-6

receptor antibodies correlated with the expression of IL-6 receptors in the implanted tissue.

In the pathology of RA, inflammatory cell infiltration is followed by progressive joint deformity and destruction with time. Blocking the action of IL-6 is an important means of treating RA to prevent the activity and infiltration of inflammatory cells. Thus, RA therapy with humanized anti-IL-6 receptor antibodies may be ineffective in cases of progressive bone deformity and destruction. When radiolabeled anti-IL-6 receptor antibodies are used in SPECT imaging, the distribution of IL-6 receptors is associated with the inflammatory cell infiltration that is seen in the early stage of RA. Accordingly, imaging with the present humanized anti-IL-6 receptor antibodies appears to be useful for detecting early pathophysiological conditions and assessing the efficacy of antibody treatment as well as the prognosis of patients with RA. Furthermore, when RA lesions are not visualized on scintigraphic images using radiolabeled anti-IL-6 receptor antibodies, antibody treatment is unlikely to be effective, and alternative RA treatments should be considered. The tracer was also taken up by organs other than target sites, such as the liver. However, because such tracer uptake in the main organs and blood can be ignored, scintigraphic imaging with humanized anti-IL-6 receptor antibodies appears to be feasible for the early detection of RA lesions and in assessing the efficacy of RA treatments.

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