Biological Background Subtraction Improves Immunoscintigraphy by Subsequent Injection of Antigen

Niels Tromholt and Johan Selmer

The Department of Clinical Physiology, Hillerød County Hospital and Novo Nordisk A/S, Bagsværd, Denmark

We have developed an ^{111}In-labeled antibody for in vivo use directed against tissue plasminogen activator demonstrating focal fibrinolytic activity. However, a major problem in immunoscintigraphy is the low signal-to-noise ratio due to circulating antibody. The hepatic clearance of t-PA is very rapid. The effect of a subsequent injection of a small amount of t-PA shortly after the antibody administration to increase the blood clearance rate of the formed antigen-antibody complexes was examined in six rabbits. More than 99% of the circulating antigen-antibody complexes were eliminated by the liver within 10 min. This technique could make immunoscintigraphy a first line diagnostic tool in acute medicine including imaging of thromboembolic lesions in organs with high blood volumes such as the lungs, the heart, and the brain.

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In a recent review of radiopharmaceuticals for thrombus detection (*1*), Linda C. Knight states, "The goal is a radiotracer that binds specifically and rapidly to thrombi, and also has a rapid blood disappearance rate, permitting a clear diagnosis within few hours after injection and without interference by anticoagulants"—a goal not yet achieved.

Evidence is now accumulating that radiolabeled monoclonal antibodies can *bind specifically and rapidly* to epitopes on the thrombi. Clinical investigations until now have mainly been performed with antibodies against platelets or crosslinked fibrin, but these antibodies have limitations in the presence of anticoagulants (*2–4*). The antifibrin antibody is the most frequently-used monoclonal antibody for thrombus detection today, and is the only one commercially available.

A high-activity ratio between a process and its background (high signal-to-noise ratio) is essential for accurate thrombus visualization. Using radiolabeled antibodies, one often has to wait 24 hr before achieving an acceptably high ratio (a time delay), which is inconsistent with acute diagnosis. Research is therefore aimed at achieving a *rapid blood clearance* rate. Antiantibodies (*5*) and antibody fragments (*6*) are used to accelerate the disappearance rate of the injected radiolabeled antibodies, though with only moderate success. Tissue plasminogen activator (t-PA) is a protease involved in fibrinolysis and is used in a recombinant version in fibrinolytical treatment of acute myocardial infarction. Imaging of recent thrombi with radiolabeled monoclonal antibodies against human t-PA gives scintigraphic results comparable to those obtained by the antifibrin antibody and, in contrast to the antifibrin antibody, the results with the anti-t-PA antibody have been achieved even in the presence of anticoagulants (*7*). Liver has a high capacity for binding and eliminating t-PA (*8,9*) yielding a half-life of t-PA in human circulation of 4 min (*10*) and in rabbits of 2 min (*11*). The present study shows that administration of t-PA after the radiolabeled antibody injection results in hepatic elimination of up to 99% of the circulating radioactive t-PA-antibody complexes within 10 min. Thus, the time needed to get a scintigraphic diagnosis may potentially be reduced from 24 hr to 1 hr following injection of t-PA.

MATERIALS AND METHODS

Antibody

A murine monoclonal antibody F2 was raised against purified melanoma t-PA. This antibody reacts with kringle 2 on human t-PA. The hybridoma cell was grown in cell culture. The antibody (subclass IgG_1) containing supernatant was purified on a Protein A matrice. $F(ab')_2$ fragments were produced by digestion of the intact IgG with pepsin at an enzyme/substrate ratio of 1:100 at pH 4.3 for 16 hr at 37°C and purified on an Ultrogel AcA 44 column. The purity was monitored on silver-stained SDS-gels.

Radiolabeling

The radiolabeling of these fragments is based on the method of Richardson et al. (*12*). The $F(ab')_2$ fragment was conjugated with the cyclic anhydride of diethylene-triaminepenta-acetic acid (cDTPA) in a molar ratio of 1:4, so the average number of DTPA groups attached to each molecule was about 1.5. The protein solution was purified by centrifugal ultrafiltration using 0.375 M acetate buffer, pH 6.0. The solution was passed through a 0.22 μM millipore filter, dispensed in vials each containing 1.1 mg and stored at–18°C. To provide a specific radioactivity on 100

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For reprints contact: Niels Tromholt, MD, Dept. of Clin. Physiol. Nucl. Med. KF 4011, Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen Ø, Denmark.

MBq/mg, 130 MBq Indium-11 chloride (Amersham) was added directly to the thawn-out protein solution. The product was made isotonic by adding 0.013 M phosphate buffer pH 7.4 containing DTPA (to remove potentially free ^{111}In ions) and passed through a 0.22 μM millipore filter. Radiochemical purity (radiolabeled protein fraction) was determined on high-performance liquid chromatography. The immunochemical purity of the conjugate was determined as the percentage of radioactivity absorbed on a t-PA coupled Sepharose 4B column.

Procedures

Six rabbits (New Zealand white) were injected with the monoclonal antibody, at 14 μg/kg per rabbit. The specific radioactivity of the antibody was 100 MBq/mg (a value which corresponds to that in our human study). The care of the rabbits was according to our institutional guidelines. Rabbit 1 and 2 received antibody at t = 0 min with no subsequent t-PA injection. Rabbit 3 received antibody mixed in vitro with t-PA in 3 × molar concentration of the antibody. Rabbits 4 to 6 received antibody at t = 0 min. At 120 min after the antibody injection these rabbits received an intravenous injection of t-PA (Actilyse, Boehringer Ingelheim) over a period of one min. The amount of t-PA injected was on a molar basis 3, 6, or 12 times the amount of the antibody injected.

Blood Samples

Blood samples were drawn at t = 0, 10, 20, 40, and 60 min and in addition to 2, 4, 6, and 12 hr after injection of antibody. In rabbits 4–6 additional samples were drawn 10, 20, 40, and 60 min after injection of t-PA. In each sample whole plasma activity and the immunoreactive activity of the radiolabeled antibody in plasma (determined as activity in plasma absorbed on a t-PA coupled sepharose column) was measured. With regression analysis (Freelance Plus ver 3.01, Lotus software) these data, corrected for physical decay of the radioactivity, were fit to an exponential curve and the half-life was calculated.

Organ Distribution

Rabbits were killed after 24 hr. Liver, kidneys, spleen, lungs, and heart were weighed and samples were counted in a gamma-wellcounter (Packard Cobra). Total activity-per-organ was calculated.

Patient Examination

One patient with symptoms of deep venous thrombosis in the right crural region was examined. The diagnosis was confirmed by phlebography prior to the immunoscintigraphy. The patient was on anticoagulant therapy (phenprocoumon and heparin) at the time of scintigraphy. A murine monoclonal antibody Line C raised against purified melanoma t-PA, and previously used in a human trial (*7*) was used. This antibody reacts with kringle 1 on human t-PA. One hr after the injection of 100 MBq ^{111}In-labeled monoclonal antibody, 5 mg t-PA (Actilyse, Boehringer Ingelheim) was injected intravenously. Scintigrams were obtained prior to and 20 min after the t-PA injection. Counts-per-pixel were determined in the area of the known thrombus and in a similar area in the contralateral leg.

RESULTS

Radiolabeling

The radiochemical purity of the antibody used in the rabbit study was 93% (Fig. 1), and the immunochemical purity was 91%. Radiochemical purity of the antibody used in the patient study was 67%.

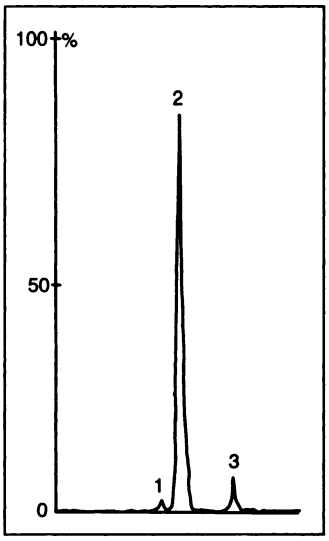

FIGURE 1. HPLC size chromatography of radiolabeled conjugate. Peak heights represent radioactivity. Peak 1 represents polymers of F(ab')$_2$, peak 2 (91%) represents F(ab')$_2$ and peak 3 free indium-DTPA.

Blood Samples

The calculated half-life for total radioactivity in plasma was 10.3 hr when no t-PA was injected. By injection of t-PA two hr after the radiolabeled antibody injection a drop in *total plasma activity* of the radiolabeled antibody was seen. Within 10 min a reduction in total plasma activity of 86%–92% was observed, a reduction independent of the t-PA dose given (Fig. 2). The decline in *immunoreactive antibody radioactivity* showed a removal of more than 99% of the radioactivity within 10 min after injection of t-PA. The half-life of this t-PA dependent elimination is 2 min (Fig. 3). A slight rise in activity—up to 3%—was seen in the hours after the initial elimination.

Values from the rabbit receiving t-PA mixed with antibody in vitro are not shown on the figures because the

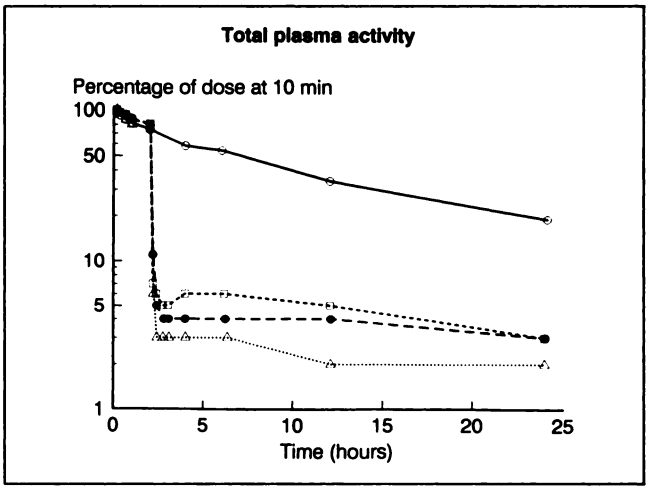

FIGURE 2. Total radioactivity in plasma as percentage of initial activity. The symbols represent: (○) antibody alone; (□), (△), (●) antibody followed by subsequent t-PA injection after 2 hr in 3, 6, and 12 times the molar amount of antibody respectively.

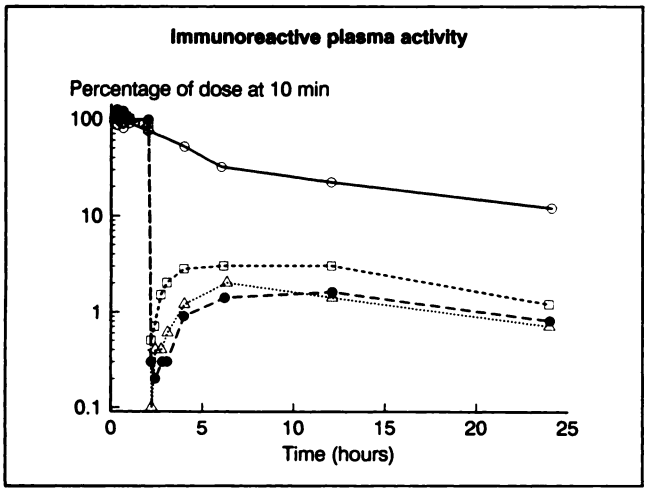

FIGURE 3. Immunoreactive radioactivity in plasma as percentage of initial immunoreactive radioactivity. The symbols represent: (○) antibody alone; (□), (△), (●) antibody followed by subsequent t-PA injection after 2 hr in 3, 6 and, 12 times the molar amount of antibody respectively.

radioactivity was eliminated even before the first blood sample was drawn.

Organ Distribution

In rabbits receiving only the radiolabeled antibody without additional t-PA the radioactivity pools were mainly located in the kidneys (20%) and blood (19%), whereas in rabbits receiving t-PA as well, up to 97% of the total activity (rabbit 3) were contained in the liver.

Patient Examination

Scintigram obtained prior to the t-PA injection did not reveal the exact location of the thrombus (Fig. 4). Increased blood-pool activity in the right leg due to congestion in the peripheral veins was detected. Twenty min after the t-PA injection, blood-pool activity was reduced and the location of the thrombus could be determined. An increase in thrombus/blood count ratio from 1.2 to 1.6 was calculated.

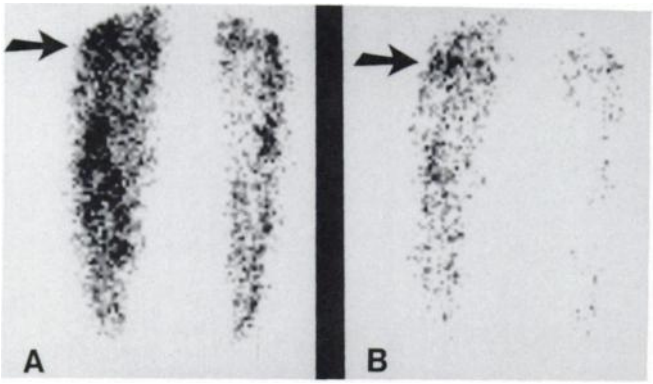

FIGURE 4. A patient with a deep venous thrombosis in the proximal crural region of the right leg (arrow). Scintigrams obtained 60 min (A) and 80 min (B) after a radiolabeled t-PA specific antibody injection. t-PA was injected immediately after the A scintigram was obtained.

DISCUSSION

The decline of total radioactivity in plasma is a parameter of great significance for imaging, since the total plasma activity will determine the target/blood ratio. The reduction in immunoreactive antibody radioactivity following t-PA injection is a measure for the in vivo efficiency of the receptor elimination pathway. The decrease in total radioactivity will be less than the reduction in immunoreactive antibody radioactivity unless the conjugate is 100% immunochemical reactive. The slight rise in activity seen in the hours after the initial elimination represent redistribution of the immunoreactive antibody from the extracellular space (*11*). The present investigation confirms that a complex between t-PA and the radiolabeled anti-t-PA antibody have a substantially shorter half-life in the circulation than the radiolabeled antibody alone. Furthermore, the experiments demonstrate that it is possible to exploit the rapid liver clearance of t-PA in vivo. There are at least two pathways by which t-PA is eliminated in the liver (*13*). The antibody used in the rabbit study reacts with an epitope on human t-PA not involved in the hepatic clearance of t-PA (*9*). The study also demonstrated that there is a difference between the decrease in total plasma radioactivity and in immunoreactive radioactivity. This difference is explained by the immunochemical impurity and underlines the importance of a high immunochemical purity of the conjugate used.

A preliminary observation in one patient has indicated that it is also possible to obtain a decrease in blood-pool activity in humans following t-PA injection without a similar reduction in activity located on the thrombus resulting in a substantial improvement in target/blood ratio.

CONCLUSION

Biological background subtraction performed by injection of a subtherapeutical dose of t-PA within 1–2 hr after antibody administration is capable of eliminating up to 99% of the background activity. This possibility of increasing the target/blood ratio opens new perspectives for the application of immunoscintigraphy as a first line diagnostic tool in acute medicine including thromboembolic lesions in organs with high blood-volume such as the lungs, heart, and brain.

It is conceivable that the use of biological background subtraction is not limited to the field of anti-t-PA imaging. Using bispecific antibodies, reactive with t-PA on one side and any other epitope on the other, our method may presumably be applied to many other forms of immunoscintigraphy.

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EDITORIAL

Immunoscintigraphy of Thrombi

Noninvasive detection and localization of thrombi remains a major challenge in clinical diagnosis. To date, clinical investigations have mainly been carried out with radiolabeled monoclonal antibodies (Mabs), which are either specific for platelets or fibrin. In order to be clinically useful for immunoscintigraphy of thrombi, such Mabs should fulfill the following criteria:

1. They should bind with high affinity to an epitope which occurs at a high density at the thrombus surface.
2. The epitope should be highly or totally thrombus-specific.
3. Epitope exposure should be independent of thrombus age and composition.
4. Antibody binding should not be affected by anticoagulant or antiplatelet drugs.
5. The antibody should not be affected by radioisotope labeling procedures.

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For reprints contact: P. Holvoet, Center for Thrombosis and Vascular Research, University of Leuven, Campus Gasthuisberg, O and N, Herestraat 49, B-3000 Leuven, Belgium.

The first report on successful thrombus radioimmunoimaging was published in 1985 (*1*). The 7E3 antibody enabled imaging of experimental venous and arterial thrombi 1.5–2.0 hr after injection and thus fulfilled the first criterion of a clinically useful antibody-based thrombus-specific agent (*2*). Binding of 7E3 to the platelet IIb/IIIa glycoprotein complex in the thrombus resulted in thrombus-to-blood ratios as high as 15 at 60–90 min after injection of the radiolabel, as a result of rapid accumulation of ^{99m}Tc-labeled 7E3 at the thrombus surface and a surprisingly rapid clearance of radiolabeled antibodies with an effective half-life of 20 min. However, because of its extensive cross-reactivity with circulating unactivated platelets, 7E3 does not fulfil the second criterion of an optimal antibody-based thrombus-specific agent. Furthermore, accumulation of 7E3 occurred only at the surface of growing thrombi, whereas maturation of thrombi resulted in the disappearance of the binding sites for 7E3. Consequently, only 1-day-old thrombi could be imaged. Finally, the binding of 7E3 to thrombi was greatly hampered in the presence of anticoagulant agents.

More recently, Mabs specific for activated platelets have been produced. Iodine-131-labeled KC_4, a Mab directed against an epitope in PAD-GEM (platelet activation-dependent granule-external membrane protein) (*3*) allowed detection of deep venous thrombi in femoral veins of baboons at 1 hr postinjection. Recently, ligand-induced binding site-specific antibodies (LIBS) have been described (*4*), which are specific for epitopes in the glycoprotein IIb/IIIa complex after its interaction with Arg-Gly-Asp-containing ligands such as fibrinogen or vitronectin. Such antibodies should discriminate between resting and activated platelets, but data on their use for thrombus imaging are presently not available.