Thrombus Imaging: A Comparison of Radiolabeled GC4 and T2G1s Fibrin-Specific Monoclonal Antibodies

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Radioimmunoimaging of experimentally-induced canine thrombi has previously been achieved with iodine-131- and indium-111-labeled (^{131}I and ^{111}In) anti-fibrin T2G1s monoclonal antibody (MAb). We now compare T2G1s to another anti-fibrin MAb, designated GC4, for imaging fresh and aged canine thrombi. GC4 is specific for a neoepitope exposed on fibrin later in the thrombolytic process after plasmin digestion. Femoral venous thrombi were induced in six groups of dogs, each containing three dogs. In two groups, the MAbs were compared when the thrombi were 3-hr or 3-days old at the time of injection, and the dogs were killed at 48 hr. In thrombi 3-hr-old, the GC4/T2G1s concentration ratio averaged 0.53 compared to 1.9 in 3-day-old thrombi. Two groups of dogs with thrombi 1- or 3-days-old were heparinized before MAb injection and were killed at 24 hr. The heparinized dogs with thrombi 1- or 3-days-old had GC4/T2G1s mean ratios of 2.3 and 2.9, respectively. In the unheparinized groups, the corresponding ratios were 1.1 and 1.9. GC4 may be more useful for clinical thrombus imaging than T2G1s because spontaneous venous thrombi are usually several days old at the time of presentation and patients are often heparinized immediately.

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Thrombus imaging, with radiolabeled fibrin-specific or platelet-specific MAbs, has been actively investigated (*1,2*). Oster et al. have successfully imaged fresh canine thrombi using a indium-111-(^{111}In) labeled MAb (7E3) specific for the glycoprotein complex IIb/IIIa antigen present on platelet membranes (*3*). Peters et al., using another platelet-specific MAb (P256) labeled with ^{111}In, had positive images with fresh (<24 hr old) human thrombi; however, the images of a single patient with an older thrombus were negative (*4*). Som et al. have used a technetium-99m-labeled Fab' fragment of a MAb (50H.19) (^{99m}Tc-50H.19) that cross reacts with platelets, for imaging fresh canine thrombi (*5*). A MAb specific for an antigen expressed on activated platelets (PADGEM) also has demonstrated sufficient localization in fresh experimental thrombi in the baboon for successful imaging (*6*).

For imaging older thrombi, fibrin-specific MAbs have been investigated. Knight et al. have imaged rabbit and dog thrombi with ^{111}In-labeled Fab fragment of a fibrin-specific MAb (59D8) (*7*). We have used a similar fibrin-specific MAb, designated T2G1s, and found that both iodine-131- (^{131}I) and ^{111}In-labeled T2G1s and its F(ab')$_2$ fragments yielded positive images in both fresh and aged canine thrombi (*8–10*). Recently, Knight et al. have reported successful imaging of canine thrombi with ^{99m}Tc-labeled Fab' fragment of T2G1s (*11*).

Anti-fibrin T2G1s MAb is specific for an antigenic site present on the beta chain (β15–21) of human fibrin which is exposed early after thrombin digestion (*12*). This site is cleaved early during fibrinolysis; consequently, antigen concentration may decrease in older thrombi. However, our results with different aged canine thrombi (1–5 days old) indicated that the percent of T2G1s localized per gram thrombus remained relatively constant and was sufficient for successful imaging (*9*). We now compare another antifibrin MAb, designated GC4, to T2G1s for thrombus imaging. GC4 is specific for a neoepitope exposed on fragment D of human and dog fibrin after plasmin digestion (*13*). GC4 binds to both the fibrin monomer and cross-linked fibrin (as well as plasmin digested fibrinogen). Radiolabeled GC4 may be advantageous for thrombus imaging due to its affinity for older venous thrombi which are typically seen clinically.

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MATERIALS AND METHODS

Monoclonal Antibody Purification

Ascites, containing T2G1s or GC4 (both are of the IgG1 subclass), was supplied by Bohdan Kudryk of the New York Blood Center. T2G1s and GC4 were purified by mixed-mode ion-exchange chromatography using Baker ABx silica based gel (J.T. Baker, Phillipsburg, NJ). A Pharmacia C 16/20 column was used with Pharmacia's FPLC system (Pharmacia Inc., Piscataway, NJ). For each run, 10 ml of ascites were diluted with 30 ml of 25 m*M* MES, pH 5.6. Ten milliters of this mixture were centrifuged at 4,000xG for 45 min and the supernatant applied to the column using a 10-ml superloop applicator at a flow rate of 1 ml/min of 25 m*M* MES buffer. After the break-through peak was collected, the antibodies were eluted by applying a 30-ml, 0–100% gradient of 1 *M* NaAc, pH 7.0, at a flow rate of 1 ml/min. The antibodies were further purified by passage through a Pharmacia superose 12 gel filtration column at a flow rate of 0.2 ml/min in 0.05 *M* Tris/0.15 MNaCl, pH 7.0, buffer.

Monoclonal Antibody Radiolabeling

MAbs were radiolabeled with ^{125}I or ^{131}I using the Pierce iodobead method (Pierce, Rockford, IL). One-hundred fifty micrograms of antibody in ~0.05 ml .05 *M* Tris/0.15 *M* NaCl, pH 7.0, were added to 150 μl of 0.2 *M* phosphate buffer, pH 7.0, followed by either ~300 μCi of ^{125}I or ~900 μCi ^{131}I and three iodobeads. After 7 min, the samples were pipetted off and free iodide removed by ultrafiltration using a C-30 centricon filter (Amicon, Danvers, MA) or with a Bio Rad P-10 desalting column (Bio Rad, Richmond, CA). The percent yield for iodination ranged from 60% to 80% of added iodine bound to antibody. Free iodide was removed by ultracentrifugation using C-30 centricon filters. For the few injected samples which contained > 10% free iodide as determined by TCA precipitation and instant thin layer chromatography (ITLC) the radiobioassay data were normalized to bound activity.

For ^{111}In labeling, 9 mg of GC4 in 9 ml of 0.42 *M* HEPES buffer, pH 7.4, were incubated with 6 mg diethylenetriaminepentaacetic acid (DTPA) cyclic dianhydride for 2 hr at room temperature. Residual free DTPA was removed and the buffer exchanged to 0.1 *M* citrate, pH 6.0, by C-30 ultrafiltration.

The amount of DTPA bound to GC4 was quantified by a binding assay in which DTPA coupled GC4 was mixed with $InCl_3$ from a 10-ml 24 μ*M* $InCl_3$/0.1 *M* citrate pH 6.0 solution containing a tracer amount of ~70 μCi ^{111}In. Ten and 20 μg (0.67 and 1.3 x 10^{-10} moles) of DTPA coupled GC4 were applied to centricon C-30 filters. After washing, 5.3 x 10^{-9} moles of $InCl_3$ were mixed in the C-30 conical collection tubes for 45 min at room temperature. Two hundred microliters of a 10-mg/ml DTPA solution (~5 x 10^{-6} moles) in citrate buffer were then added to chelate free ^{111}In. Two milliliters of citrate buffer were added and the samples were centrifuged, washed twice with 2.0 ml citrate buffer, and the filter counted in a gamma counter. The molar amounts of bound ^{111}In were determined by dividing the retained counts by the specific activity of the $InCl_3$ solution. The assay indicated that six DTPA chelating groups were coupled per molecule of GC4. Previous results showed that GC4 coupled with higher amounts of DTPA exhibited a decrease in immunoreactivity (*15*).

For imaging experiments, ^{111}In-citrate was prepared from commercial carrier-free indium chloride in 0.05 *M* HCl by adding an equal volume of 0.1 *M* sodium citrate buffer, pH 6.0. One hundred and fifty micrograms of DTPA-GC4 in 39 μl were mixed with ~800 μCi of ^{111}In-citrate for 30 min. The final sample contained <3% unbound ^{111}In as estimated by ITLC.

Immunoreactivity Determination

The immunoreactivity of radiolabeled T2G1s was measured by affinity chromatography in which human fibrin was covalently bound to Pharmacia sepharose 6MB as previously described (*14*). To determine GC4 immunoreactivity, fibrinogen was digested with plasmin and the digested mixture coupled to CNBr-activated sepharose 6MB as previously described (*15*). Maximum immunoreactivity for both T2G1s and GC4 was determined by double reciprocal plots of total/bound radioactivity versus weight of antigen-gel per tube as described by Lindmo et al. (*16*). To determine if GC4 and T2G1s binding was competitive, 50 ng of ^{111}In-GC4 and 0.2 ml plasmin digested fibrinogen-coated gel were incubated alone or with 40 μg of cold GC4, T2G1s, or control nonspecific murine IgG. Percentage GC4 binding was determined by dividing the gel-bound counts, after two washes with 0.05 *M* Tris/0.15 *M* NaCl containing 0.1% BSA, by the total counts added per tube.

Imaging

Thrombi were produced by transcatheter placement of a Gianturco coil (Cook, Bedford, MA) into a femoral vein of mongrel dogs (*17*). Coil position was confirmed by fluoroscopy and radiography. Some dogs underwent heparin therapy initiated 3 hr prior to antibody injection and continued throughout the duration of the experiment. Bovine heparin 350–500 units per kg were injected subcutaneously three times a day.

Each dog was injected intravenously in a forelimb with ~50 μg of T2G1s and GC4 from 3 hr to 3 days after thrombus induction. The injected activities used were ~250 μCi of ^{131}I, ~50 μCi of ^{125}I, or ~300 μCi ^{111}In. Anterior images using a Ohio Nuclear series 100 gamma camera of the lower pelvis and legs, abdomin and chest were obtained immediately and at 1–4, 24, and in some dogs at 48 hr. Images of dogs injected with ^{111}In-GC4 were computer generated. After the last image, 50 cc of Renografin-60 were injected into a dorsal vein of the foot to obtain a venogram for comparison.

Blood was drawn at 5, 10, 20, 30, 45, and 60 min, and at 2, 3, 4, 24, and 48 hr after antibody injection. After the dogs were killed, the amounts of radiolabeled GC4 and T2G1s were measured in all major organs and thrombus in which blood and normal muscle and the results were expressed as "% of injected dose per gram of tissue" as determined by dual-channel gamma counting.

RESULTS

Immunoreactivity and Binding Studies

Immunoreactivity of radiolabeled T2G1s and GC4 was determined by affinity chromatography using fibrin-coupled sepharose or plasmin digested fibrinogen-coupled sepharose. Maximum antibody binding at in-

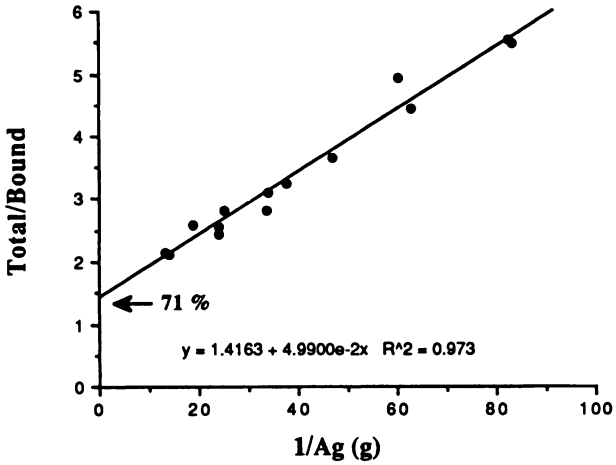

FIGURE 1
Immunoreactivity of ^{111}In-labeled GC4 was determined by a double reciprocal plot of total/bound radioactivity versus weight of antigen-coupled sepharose. Extrapolation to the ordinate indicated 71% immunoreactivity.

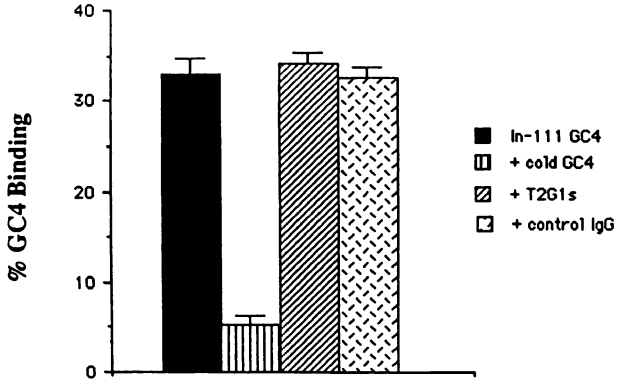

FIGURE 2
Affinity chromatography with antigen-coupled sepharose and ^{111}In-GC4 was performed to characterize GC4 binding. Indium-111-GC4 added alone and with excess cold GC4, T2G1s or nonspecific murine IgG, indicated specific and saturable binding and no competition with T2G1s or nonspecific IgG.

finite antigen excess was estimated by a double reciprocal plot of antibody binding versus gel weight. Shown in Figure 1 is the immunoreactivity determination for ^{111}In-GC4. Both MAbs were determined to be >70% immunoreactive. To demonstrate that GC4 and T2G1s binding is not competitive, affinity chromatography with plasmin digested fibrinogen-coupled sepharose gel was used (Figure 2). Indium-111-GC4 bound ~33% after incubation with 0.2 ml of antigen-coupled gel. When 40 μg of cold GC4 were added to the mixture, the binding dropped to ~5%. When 40 μg of nonspecific murine IgG or T2G1s were added, there was no decrease in GC4 binding. Thus, GC4 exhibits specific and saturable binding with the antigen gel and T2G1s and GC4 bind to different antigenic sites on fibrin.

Imaging

Composite venograms and 4-, 24-, and 48-hr nuclear images of ^{131}I-GC4 in dogs with 3-hr-old thrombi are shown in Figure 3A and 3-day-old thrombi in Figure 3B. In all dogs, the 24- and 48-hr images after antibody injection were clearly positive. Each venogram showed a filling defect corresponding to the site of ^{131}I-GC4 accumulation as denoted by the nuclear images.

In Figure 4A, ^{131}I-T2G1s images and a venogram are shown in a heparinized dog with a 1-day-old thrombus. Both the 4- and 24-hr images were positive in these dogs. Similar images are shown in Figure 4B with ^{131}I-GC4 in heparinized dogs containing 3-day-old thrombi. Thus, both T2G1s and GC4 achieve adequate localization and thrombus/blood and thrombus/muscle ratios producing positive images in heparinized dogs.

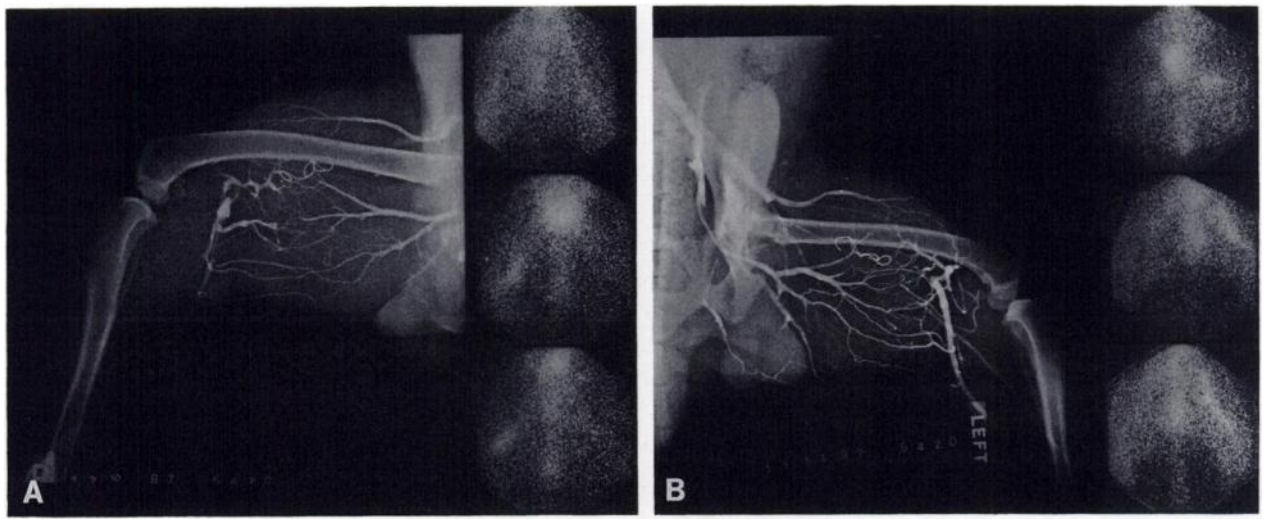

FIGURE 3
Venograms and 4-, 24-, and 48-hr anterior images of dogs with experimentally-induced femoral vein thrombi after injection of ^{131}I-labeled GC4. Thrombi were aged 3 hr (A) and 3 days (B) before MAb injection.

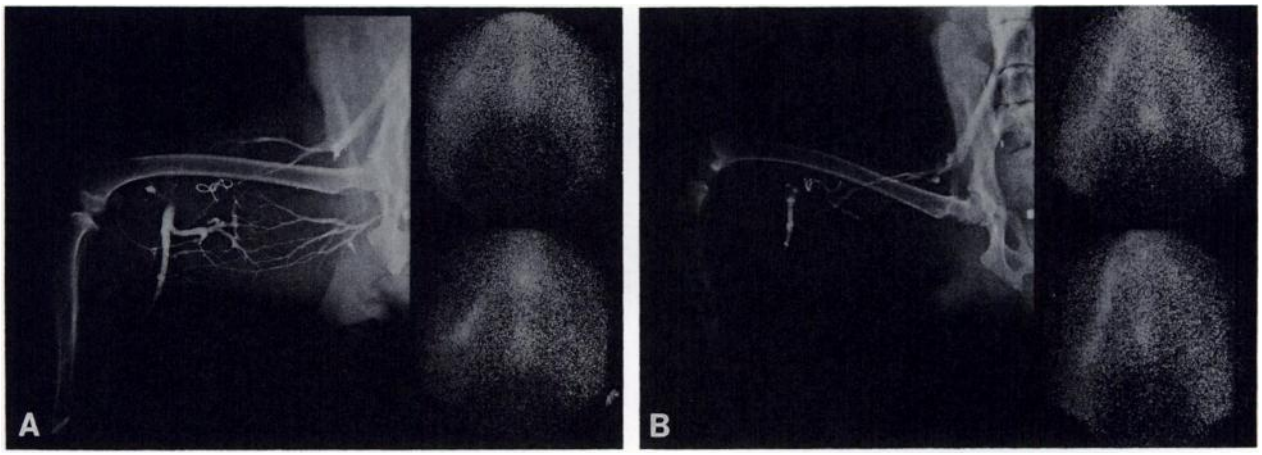

FIGURE 4
Venograms and 4- and 24-hr anterior images of heparinized dogs with (A) a 1-day-old thrombus when injected with ^{131}I-T2G1s and (B) a 3-day-old thrombus when injected with ^{131}I-GC4.

Four- and 24-hr images of ^{111}In-GC4 are shown in Figure 5A of a dog with a 1-day-old thrombus. The early images were suggestive but the 24-hr images were clearly positive. Figure 5B is a composite of 4- and 24-hr images in a heparinized dog with a 3-day-old thrombus. Both the 4- and 24-hr images were positive. Figure 6 is a composite of sequential images taken from a heparinized dog containing a 3-day-old thrombus and injected with ^{111}In-GC4. The images were positive 3 hr after ^{111}In-GC4 injection, with noted asymmetry between the thrombosed leg and the contralateral normal leg.

Radiobioassay and Blood Clearance

Six groups of dogs with induced femoral venous thrombi were studied, each containing three dogs. The amount of GC4 and T2G1s was measured in each dog in major organs and tissues, including thrombus, blood, and normal muscle. Except for the thrombus differences, GC4's distribution in vivo was similar to previously reported values for T2G1s (*10*). Data were averaged and listed in Tables 1–3 as percentage of injected dose per gram of tissue. In dogs with 3-hr-old thrombi (Table 1, top), the thrombus ^{131}I-GC4/^{125}I-T2G1s ratio was 0.53 and the clot/blood and clot/muscle ratios were 9 and 120 for GC4 and 9 and 178 for T2G1s. In dogs with 3-day-old thrombi (Table 1, bottom) the thrombus ^{131}I-GC4/^{125}I-T2G1s ratio was 1.9 and the clot/blood and clot/muscle ratios were 12 and 176 for GC4 and 5 and 87 for T2G1s.

Blood clearance is plotted in Figure 7 for ^{131}I-GC4 and ^{125}I-T2G1s in unheparinized dogs (n=6). For both antibodies, the blood clearance was best fit by a biphasic plot. GC4 cleared faster than T2G1s in each phase resulting in better images due to higher thrombus/blood ratios. The blood clearance of ^{111}In-GC4 was similar to ^{131}I-GC4; however, as commonly found with radioimmunoimaging studies using ^{111}In-labeled MAbs, liver concentrations of ^{111}In were high (~30% of dose) and transchelation by transferrin and bone marrow sequestration occurred.

The concentrations of GC4 and T2G1s in thrombi

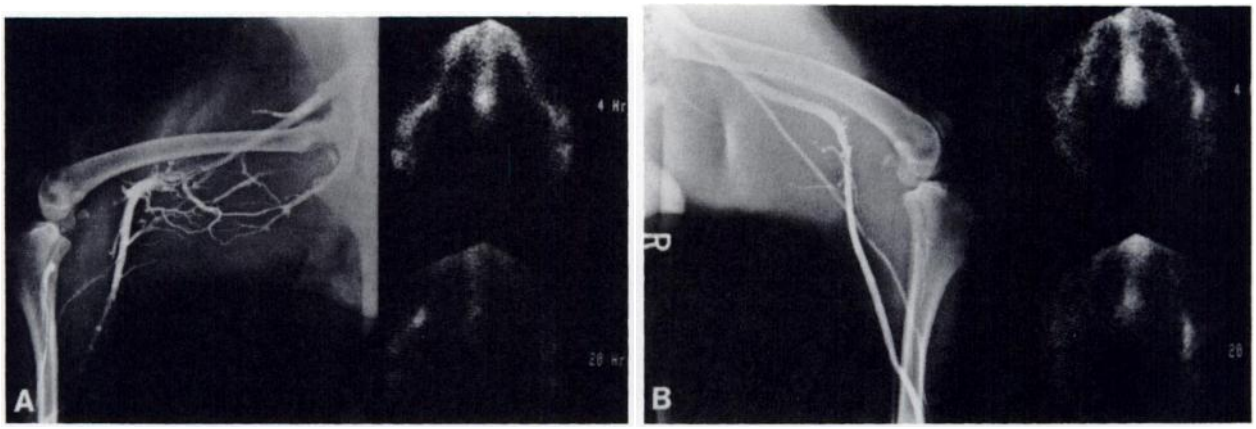

FIGURE 5
Venograms and 4- and 24-hr anterior images of (A) a dog with a 1-day-old thrombus and (B) a heparinized dog with a 3-day-old thrombus when injected with ^{111}In-GC4.

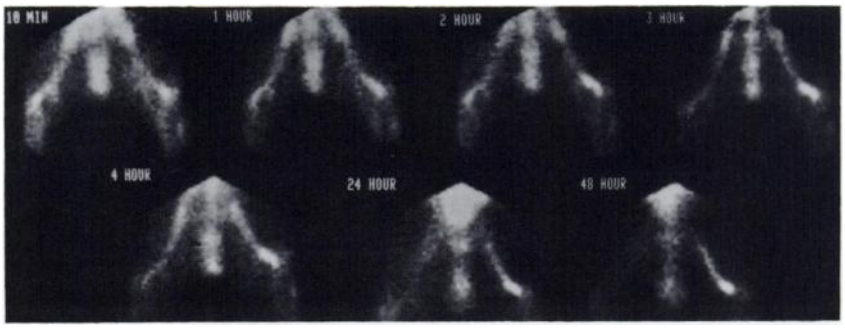

FIGURE 6
Images taken from 10 min to 48 hr of a heparinized dog containing a 3-day-old thrombus show positive images 3–48 hr after ^{111}In-GC4 injection.

of dogs undergoing heparin therapy, and killed at 24 hr after antibody injection, are listed in Table 2. In dogs with 1-day-old thrombi the amount of ^{125}I-GC4 in the thrombus (0.534% dose per gram) was striking (Table 2, top). The ^{125}I-GC4/^{131}I-T2G1s clot ratio was 2.3 and the clot/blood and clot/muscle ratios were 24 and 557 for GC4 and 9 and 265 for T2G1s. Similar results were found in dogs with 3-day-old thrombi (Table 2, bottom). The ^{131}I-GC4/^{125}I-T2G1s clot ratio was 2.9, and clot/blood and clot/muscle ratios were 21 and 573 for GC4 and 6 and 146 for T2G1s.

Radiobioassay data (Table 3, top) from dogs with 1-day-old clots and injected with ^{111}In-GC4 and ^{125}I-T2G1s had a similar localization of the two MAbs. However, allowing the thrombus to age 3 days before MAb injection and treating the dog with heparin increased the thrombus concentration of ^{111}In-GC4 and the GC4/T2G1s ratios (Table 3, bottom). In this group, one dog had exceptionally high concentrations of both GC4 and T2G1s, which resulted in a large standard deviation.

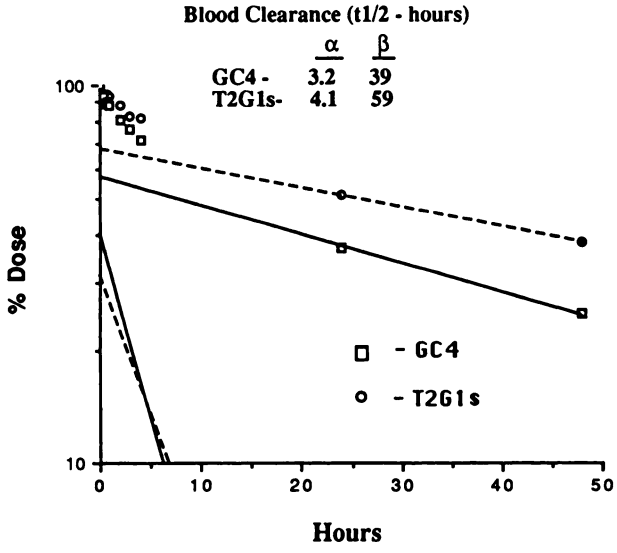

FIGURE 7
Blood clearance of ^{131}I-GC4 (solid line) and ^{125}I-T2G1s (dashed line). The data for both MAbs were best fit by a biexponential function, ^{131}I-GC4 cleared faster in both phases.

DISCUSSION

Radiolabeled fibrin-specific MAbs show promise for thrombus imaging because of their specificity, homogeneity, and high avidity. Other radiolabeled blood components participating in the coagulation response, e.g., platelets, plasmin, fibrinogen, and fibrin-split products, have not been adopted clinically due to their low thrombus uptake and/or their affinity for only fresh or growing thrombi (*1,2*). MAbs specific for some of these components have had similar limitations and results, i.e., antibodies specific for fibrinogen (*18*), platelets (*3–5*), activated platelets (*6*), and thrombospondin (*1*).

In this study, we compared two fibrin-specific MAbs, T2G1s and GC4, in fresh and aged canine thrombi. T2G1s and GC4 retained considerable immunoreactivity after DTPA coupling or iodination (>70%). However, the immunoreactivity of GC4 was decreased by excessive DTPA coupling, so that immunoreactivity was inversely related to the quantity of bound DTPA (*15*). The target site of T2G1s is cleaved from fibrin polymer early during plasmin digestion; this may be a disadvantage for clinical imaging because thrombi are often several days old when symptomatic. In contrast, GC4 is specific for a neoepitope exposed on fibrin later during plasmin digestion and therefore may be more appropriate to the usual clinical situation.

GC4 binding was higher than T2G1s in older thrombi, and the GC4/T2G1s ratio increased after heparinization. We currently have no definitive explanation for the heparin augmentation. Theoretically, one may argue that heparin prevents further fibrin formation and deposition and because GC4 binds to an antigenic site exposed during fibrin degradation (plasmin lysis), the addition of heparin should increase antigen concentration and prevent "covering" or "blanketing" of the GC4 target. In any event, the positive effect of heparin is welcome in the practical arena of thrombus detection because many (if not most) patients are heparinized by the time of the imaging studies.

GC4 studies of 1-day-old thrombi, were clearly positive at 24 hr, but only suggestive at 4 hr. In heparinized dogs with 3-day-old thrombi, however, GC4 localization was 1.9 times higher than that of T2G1s and the

TABLE 1
GC4 and T2G1s Localization in 3-Hour and 3-Day-Old Thrombi at 48-Hour Sacrifice

Sample	3-hr thrombi ^{131}I-GC4	^{125}I-T2G1s	GC4/T2G1s
*Clot	0.091 ± 0.030	0.172 ± 0.057	0.53
*Blood	0.11 ± 0.002	0.019 ± 0.004	0.58
*Muscle	0.0008 ± 0.0004	0.0011 ± 0.0008	0.73
Clot/blood	9.0 ± 4.4	9.4 ± 3.8	0.96
Clot/muscle	120 ± 37	178 ± 81	0.67
	Thrombus weight = 0.703 ± 0.137 g		
Sample	3-Day thrombi ^{131}I-GC4	^{125}I-T2G1s	GC4/T2G1s
*Clot	0.190 ± 0.162	0.103 ± 0.073	1.9
*Blood	0.017 ± 0.002	0.025 ± 0.006	0.68
*Muscle	0.0010 ± 0.0003	0.0011 ± 0.0003	0.91
Clot/blood	11.9 ± 11.5	4.5 ± 4.1	2.6
Clot/muscle	176 ± 100	87 ± 44	2.0
	Thrombus weight = 0.376 ± 0.156 g		

* = % dose per gram.

thrombus-to-background ratios were ~2.5 greater. In these dogs, both the 4-hr and 24-hr images were positive. Thus, the elusive goal of imaging the usual type of thrombus (several days old) in the usual circumstance (heparinized) within a reasonable period of time (4 hr) may be close to reality.

The present means of prevention and treatment of thrombolytic disorders are still limited. Considerable research is now in progress using different approaches, including inhibition of coagulation factors or platelet function, thrombolytic agents, and stimulation of the profibrinolytic properties of endothelium (*19*). Several MAbs specific for human fibrin cross react with fibrin of certain animal species (including T2G1s and GC4) and they should become valuable research tools for assessing the efficiency of thrombolytic drugs both experimentally and clinically.

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TABLE 2
GC4 and T2G1s Localization with Heparinization in 1- and 3-Day-Old Thrombi at 24-Hour Sacrifice

Sample	1-Day thrombi ^{125}I-GC4	^{131}I-T2G1s	GC4/T2G1s
*Clot	0.534 ± 0.242	0.233 ± 0.070	2.3
*Blood	0.023 ± 0.003	0.027 ± 0.004	0.85
*Muscle	0.0010 ± 0.0002	0.0009 ± 0.0002	1.1
Clot/blood	24.3 ± 14.1	8.8 ± 2.9	2.8
Clot/muscle	557 ± 201	265 ± 47	2.1
	Thrombus weight = 0.525 ± 0.152 g		
Sample	3-Day thrombi ^{131}I-GC4	^{125}I-T2G1s	GC4/T2G1s
*Clot	0.548 ± 0.202	0.190 ± 0.037	2.9
*Blood	0.029 ± 0.009	0.036 ± 0.008	0.81
*Muscle	0.0012 ± 0.0006	0.0014 ± 0.0004	0.86
Clot/blood	21.0 ± 11.3	5.5 ± 1.2	3.8
Clot/muscle	573 ± 466	146 ± 75	3.9
	Thrombus weight = 0.603 + 0.198 g		

* = % dose per gram.

TABLE 3
GC4 and T2G1s Localization in 1-Day-Old Thrombi and in Heparinized 3-Day-Old Thrombi at 24-Hour Sacrifice

Sample	1-Day thrombi		
	^{111}In-GC4	^{125}I-T2G1s	GC4/T2G1s
*Clot	0.221 ± 0.104	0.194 ± 0.039	1.1
*Blood	0.023 ± 0.002	0.032 ± 0.003	0.72
*Muscle	0.0011 ± 0.0001	0.0014 ± 0.0003	0.79
Clot/blood	9.8 ± 5.7	6.2 ± 1.9	1.6
Clot/muscle	211 ± 42	143 ± 49	1.5
	Thrombus weight = 0.247 ± 0.101 g		
Sample	3-Day thrombi		
	^{111}In-GC4	^{125}I-T2G1s	GC4/T2G1s
*Clot	0.504 ± 0.598	0.264 ± 0.210	1.9
*Blood	0.023 ± 0.004	0.033 ± 0.006	0.70
*Muscle	0.0009 ± 0.0002	0.0012 ± 0.0004	0.75
Clot/blood	24.8 ± 30.9	9.12 ± 8.7	2.7
Clot/muscle	612 ± 761	255 ± 260	2.4
	Thrombus weight = 0.322 ± 0.223 g		

* = % dose per gram.

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