Localization of Cardiac Myosin-Specific Antibody in Myocardial Infarction

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A B S T R A C T Specific localization of purified antibody against cardiac myosin has been demonstrated in areas of altered myocardial membrane permeability after experimental myocardial infarction. Intravenously administered radioiodine-labeled antimyosin was selectively localized in infarcted myocardium of seven dogs 24 h after coronary occlusion. The mean ratio (±SE) of antimyosin antibody in infarcted to normal myocardium in the center of the infarct was 4.2±0.4 for endocardial and 2.9±0.3 for epicardial layers. By utilizing (Fab')s fragments of antimyosin obtained by pepsin digestion of purified antibody, the ratio of uptake was increased in eight dogs to 6.1±0.6 in the endocardial and 3.3±0.4 in the epicardial layers at the infarct center 24 h after occlusion. These ratios were further increased in the infarct center to 13.8±1.2 in the endocardial and 7.3±0.8 in the epicardial layers when eight dogs were sacrificed 72 h after coronary occlusion.

The specificity of antimyosin (Fab')s localization in infarcted myocardium was demonstrated in four dogs by simultaneous intravenous administration of 131I-labeled antimyosin (Fab')s and 131I-labeled normal rabbit gamma globulin (Fab')s. Non-specific trapping of normal rabbit IgG (Fab')s was observed to be about 38% of total antimyosin (Fab')s uptake in the central zone of infarction.

Regional blood flow was related to antimyosin (Fab')s uptake in infarcted myocardium by utilizing simultaneous administration of 85Sr-labeled microspheres. An inverse exponential relationship between antimyosin (Fab')s uptake and regional blood flow was observed (r = 0.85). The specific localization of antimyosin antibody or its (Fab')s components in infarcted myocardium suggests a conceptually new approach to myocardial infarct localization and sizing.

INTRODUCTION

After acute myocardial infarction, there is an increase in permeability of the myocardial cell membrane, resulting from ischemic damage. This allows the egress into the plasma of intracellular macromolecular constituents such as creatine phosphokinase (1), serum glutamate oxalacetate transaminase (2), and lactate dehydrogenase (3), as well as cations such as potassium (4). This ischemia-induced increase in permeability of damaged myocardial cell membranes should also allow the inward diffusion of other macromolecules.

The purpose of this investigation was to determine whether radioiodine-labeled antibody to cardiac myosin or its (Fab')s fragments would localize specifically in infarcted myocardium in vivo. In this communication, we report the specific and selective localization of cardiac myosin-specific antibody and (Fab')s fragments in infarcted myocardium and relate this localization to the severity and duration of ischemia.

METHODS

Extraction of cardiac myosin. Cardiac myosin was isolated from canine left ventricular myocardium according to the procedure of Katz et al. (5). Myosin was further purified by ammonium sulfate precipitation (37-42% saturation) as described by Wikman-Coffelt et al. (6). The myosin obtained was solubilized in 0.5 M KCl, then dialyzed against 40 vol of 0.15 M potassium phosphate, pH 7.5, 10 mM (EDTA) for 24 h. Particulate materials were removed by centrifugation at 100,000 g for 30 min at 4°C. Homogeneity of myosin was determined by sodium dodecyl sulfate gel electrophoresis in a discontinuous buffer system as described by Neville (7), with 10% acrylamide gels. The preparation was found to have two myosin light and one myosin heavy chain bands and slight traces of actin.
Immunization procedures. Purified canine cardiac myosin (0.5 mg/ml) was emulsified in an equal volume of complete Freund's adjuvant and injected intradermally and into the toe pads of three New Zealand white rabbits by the schedule of Lawrence et al. (8). The primary immunization consisted of 500 μg of canine cardiac myosin per rabbit, followed 3 wk later by secondary immunization with 400 μg of myosin. At the 5th wk, a booster injection of 300 μg was given. 1 wk later, 50 ml of blood was obtained from each animal by ear artery incision. Weekly bleedings were subsequently obtained, and booster injections of 500 μg myosin/animal in complete Freund's adjuvant were administered intradermally monthly for 12 mo.

Determination of antibody activity. Antibodies to canine cardiac myosin were tested initially for antibody content by double immunodiffusion with 1% agarose in 50 mM potassium phosphate, pH 7.5, and 0.15 M NaCl (9). The antibody activity of purified antimyosin or its (Fab')2 fragments was determined by the binding capacity of the antibody for iodine-125-labeled canine cardiac myosin (125I-myosin) prepared according to the lactoperoxidase method of Marchalonis (10). Normal rabbit IgG (Pentex Biochemical, Kankakee, Ill.) was used as a nonimmune control. To 10-μl samples (approximately 0.5 ng/10 μl) of 125I-myosin, each containing approximately 20,000 cpm, various concentrations of antibody or (Fab')2 fragments were added. The reactants were incubated at 37°C for 1 h in 100 μl of 1% bovine serum albumin (BSA) 1 (Pentex) in 0.3 M Na phosphate, 0.15 NaCl (phosphate-buffered saline, PBS), pH 7.0. Unlabeled carrier normal rabbit IgG (Pentex) (20 μg/tube) was added, followed by 0.5 ml of 1:5 dilution of goat anti-rabbit IgG serum (Pentex, lot 16). After a 1-h incubation at 37°C, the precipitates were centrifuged for 15 min at 5,000 g at 4°C. The precipitate from each tube was washed in 1 ml of 0.3 M PBS. Radioactivities in the precipitates and supernatant solutions were determined by counting in a gamma well scintillation spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill., model 1085). Percent of 125I-myosin bound at various antimyosin (Fab')2 concentrations for a representative preparation is illustrated in Fig. 1, as calculated from the following expression: % bound = [(cpm in precipitate) × 100/(cpm in precipitate) + (cpm in supernatant solution)].

No interspecies cross-reactivity was observed between rat cardiac myosin and rabbit anticanine cardiac myosin serum. However, cross-reactivity was observed between rabbit anticanine cardiac myosin serum and canine skeletal muscle myosin. A 50% inhibition of maximum binding of 125I-labeled canine cardiac myosin by anticanine cardiac myosin serum (at 1:100 dilution) required 2.5 times more canine skeletal muscle myosin by weight than homologous canine cardiac myosin.

Purification of cardiac myosin-specific antibody. Rabbit antibody specific for canine cardiac myosin was purified by affinity chromatography with a myosin-Sepharose immunoadsorbent, prepared by coupling purified canine cardiac myosin to cyanogen bromide-activated agarose (Sepharose-4B, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) by the general method described by Cuatrecasas (11). The immunoadsorbent was equilibrated in 30 mM KCl, 25 mM Tris-HCl, pH 7.5, 1 mM EDTA (Tris-buffer).

To a 50-ml sample of rabbit anticanine cardiac myosin serum, 25 ml of saturated ammonium sulfate solution was added with constant stirring to give a final 33% saturation. The precipitate was separated by centrifugation at 10,000 g for 15 min at 4°C, then dissolved in Tris buffer and dialyzed against 40 vol of Tris buffer at 4°C for 24 h. The protein solution containing the antimyosin antibody was then chromatographed on the myosin-Sepharose immunoadsorbent. After application of the sample, the column was washed with Tris buffer until the eluate showed no absorbance at 280 nm. Myosin-specific antibody was then desorbed with 5 M guanidine-HCl. Fractions containing the antibody were pooled and then dialyzed against 40 vol of Tris-buffer at 4°C for 24 h. Small amounts of insoluble material were removed by centrifugation at 10,000 g for 30 min at 4°C. The supernatant solution was then concentrated by vacuum dialysis to approximately 2 mg/ml as determined by absorbance at 280 nm. The antimyosin antibody solution was stored at -20°C. The elution profile of a typical affinity chromatography run on 2.5 x 70-cm myosin-Sepharose column is shown in Fig. 2.

Preparation of (Fab')2 fragments. Antisera to canine cardiac myosin purified by affinity chromatography was subjected to hog pepsin (Sigma Chemical Co., St. Louis, Mo.) digestion at 37°C for 20 h at an enzyme-to-substrate concentration ratio of 1:100, according to the procedure described by Edelman and Marchalonis (12). At the end of the incubation, the reaction mixture was centrifuged at 10,000 g for 30 min. The supernatant solution was then submitted to Sephadex G-100 (Pharmacia) column chromatography (2.5 x 70 cm). Intact antimyosin antibody and protein aggregates were eluted in the void volume. The first protein peak to be eluted in the included volume, comprising approximately 80-90% of the total protein applied to the column, contained the (Fab')2 components of antimyosin antibody, concentrated by vacuum dialysis to approximately 2 mg/ml, as described previously.

(Fab')2 fragments of normal rabbit gamma globulin were also prepared as described above.
Radioiodination. Radioiodination of intact antimyosin antibody, its (Fab')2 fragments, and (Fab')3 fragments of normal rabbit IgG was performed by the lactoperoxidase procedure of Marchalonis (10). Carrier-free 125I (New England Nuclear, Boston, Mass.) was used for the iodination of intact antimyosin antibody and (Fab')3 fragments preparations. 125I, also carrier-free, was used to iodinate (Fab')2 fragments of normal IgG by the same method. Covalently bound labeled iodine was separated from free radiiodine by Sephadex G-25 gel permeation chromatography on a 0.5 × 10 cm-column precoated with 1.5 ml of 1% BSA in 0.3 M PBS to reduce nonspecific binding of radiiodinated antibody to the column matrix. Radioiodinated proteins were eluted in the void volume, and were stored at −20°C. The specific activity of radiiodinated antimyosin antibody or its (Fab')3 fragments was approximately 130 Ci/mM.

Experimental myocardial infarct model. 30 mongrel dogs (19–22 kg) were anesthetized with intravenous pentobarbital (30 mg/kg) after which a left thoracotomy was performed under sterile conditions. Acute myocardial infarction was produced by the method described by Beller et al. (13). Confluent branches of the left anterior descending coronary artery were ligated 5 min apart until approximately 30–50% of the anterolateral wall appeared cyanotic. Coronary venous branches remained intact. Any dogs developing ventricular fibrillation (10%) were not included in the study. An indwelling canula was inserted in the left atrium via a stab wound in the left atria appendage in those dogs receiving microspheres for regional blood flow determination. The thoracotomy was then closed and the animals were allowed to recover from anesthesia. All animals alive for more than 24 h received a 250-mg daily dose of ampicillin. All these animals appeared healthy and active during recovery. The left atrial catheter was flushed several times daily with heparinized saline.

Localization of whole antimyosin antibody or its (Fab')3 fragments. Radioiodinated antimyosin antibody or (Fab')3 fragments were administered intravenously 4 h after coronary occlusion in 23 dogs. Each animal received 100 μCi of intact antimyosin antibody or (Fab')3 fragments injected through a disposable Swinnex-13 filter unit (0.22 μm pore size; Millipore Corp., Bedford, Mass.), prefilled with 5 ml of 1% BSA in 0.3 M PBS. The amount of radioactive protein received by each animal was less than 100 μg 24 h after coronary occlusion, animals were sacrificed and myocardial samples (0.5–1.0 g) from epicardial and endocardial layers of the center and periphery of the infarct, the adjacent border zone region, and normal posterior left ventricular myocardium were obtained. Concentration of radioiodinated material in each sample was determined by gamma scintillation counting and counts per minute per gram wet weight of the samples were calculated. In another group of eight dogs that had received iodine-125-labeled antimyosin (Fab')3 fragments, antibody uptake in infarcted myocardium was determined 72 h after coronary occlusion as described above.

The relative antimyosin antibody or (Fab')3 concentration ([Ab1]/[Ab2]) in these samples was calculated as the ratio of 125I labeled antimyosin antibody or (Fab')3 in the infarcted myocardium (I) to that present in normal posterior left ventricular myocardium (N).

Specific localization of antimyosin (Fab')3, in infarcted myocardium. In another group of four experimentally infarcted dogs, 100 μCi each of 125I-labeled antimyosin (Fab')3 fragments and iodine-131-labeled normal rabbit IgG (Fab')3 fragments were simultaneously administered i.v. 4 h after coronary occlusion. 48–72 h later, the animals were sacrificed, and 125I and 131I activities in multiple myocardial tissue samples were determined by differential spectrometry. The specific localization of antimyosin (Fab')3 concentration and relative normal IgG (Fab')3 concentration in the same tissue sample, by the following equation: specific localization = ([Ab1]/[Ab2]) − ([IgG]/[IgG1])..

Relationship between regional myocardial blood flow and specific antimyosin (Fab')3 uptake. Regional myocardial blood flow and iodine-125-labeled antimyosin (Fab')3 uptake were simultaneously assessed in four dogs that underwent coronary occlusion for 24 h. 100 μCi of 125I-labeled antimyosin (Fab')3 were administered as described previously 15 min before sacrifice, a bolus of 4 × 106 carbogenized microspheres (15±5 μm, 3M Co., St. Paul, Minn.) labeled with strontium-85 was injected into the left atrium via the indwelling canula. Microsphere distribution and antimyosin (Fab')3 uptake were obtained by differential spectrometry in myocardial samples from infarct, border, and nonischemic zones. A similar protocol was carried out in another group of four infarcted dogs sacrificed 72 h after coronary occlusion.

Statistical analysis. Statistical differences between ischemic and nonischemic values were assessed by paired t test (14). In studies involving comparison of relative antibody uptake to regional blood flow, linear regression curves relating antibody uptake to flow were obtained by utilizing y = a + bx, where y = log10 (percent normal myocardial blood flow), and x = relative antibody uptake. Constants a and b were obtained by the method of Snedecor and Cochran (14).

RESULTS

Localization of 125I-labeled antimyosin antibody in infarcted myocardium. Relative localization of 125I-labeled antimyosin antibody in central and peripheral zones of infarction and in the border zone region in

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FIGURE 3  Antimyosin antibody binding in infarcted myocardium 24 h after coronary occlusion. Relative antibody uptake ([AbI]/[AbN]) per gram of endocardial and epicardial layers from infarct center, periphery, border zone region, and normal myocardium of seven dogs is shown. One SEM above and below the mean is depicted at the top of each bar. *P ≤ 0.001; **P < 0.01; ***P ≤ 0.05.

seven dogs after 24 h of coronary occlusion is shown in Fig. 3. In the central zone of infarction, the relative antibody uptake was 4.3±0.4 (SE) in endocardial and 2.9±0.3 in epicardial layers. These ratios were significantly greater than unity, as defined for nonischemic posterior left ventricular myocardium (P < 0.001). In the periphery of the infarct zone, the ratio of antibody uptake in this zone to normal myocardial uptake was 2.9±0.4 in endocardial and 2.0±0.2 in epicardial layers (P < 0.001 for both values). These ratios were significantly less than those observed in the central infarct zone. Endocardial uptake of antimyosin antibody was significantly greater than the epicardial uptake in both central and peripheral infarct zones.

Localization of 125I-labeled antimyosin (Fab′)2 fragments in infarcted myocardium. To improve the specific uptake of radioiodine-labeled antibody in infarcted as compared to normal myocardium, intravenous doses of 125I-labeled antimyosin (Fab′)2 fragments were administered to a group of eight dogs 4 h after coronary occlusion. Fig. 4 shows relative antimyosin (Fab′)2 uptake in myocardial samples from infarcted and border zone regions in the animals occluded for 24 h. In the infarct center, relative antimyosin (Fab′)2 uptake was 6.0±0.6 in endocardial and 3.3±0.4 in epicardial layers (P < 0.001 for both). In the infarct periphery, the ratios for antimyosin (Fab′)2 uptake were 4.4±0.4 in endocardial and 2.4±0.2 in epicardial layers (P < 0.001 for both). Border zone uptake of antimyosin (Fab′)2 was 2.7±0.5 in endocardial (P < 0.01) and 1.4±0.4 in epicardial samples (P < 0.05). Antimyosin (Fab′)2 uptake in infarcted and border zone samples was significantly greater than uptake of whole antimyosin antibody as demonstrated in the previous experiments, indicating that the initial rate of antimyosin uptake may depend on the molecular size of the antimyosin preparation employed.

The effect of a longer equilibration time on relative antimyosin (Fab′)2 uptake was investigated in another eight dogs undergoing 72 h of coronary occlusion before sacrifice. As shown in Fig. 5, the mean ratio of relative antimyosin (Fab′)2 uptake in the center of the infarct was 13.8±1.2 in endocardial (P < 0.001) and 7.3±0.8 (P < 0.001) in epicardial samples. Relative antimyosin (Fab′)2 uptake in these 72-h infarcted dogs was markedly higher than that observed for the 24-h occluded group in both infarcted and border zone regions. As previously observed in dogs receiving whole antimyosin antibody, antimyosin (Fab′)2 fragments localized significantly more in subendocardial than in subepicardial layers.

Comparison of specific localization of antimyosin (Fab′)2 components and nonspecific trapping of normal IgG (Fab′)2, fragments. Fig. 6 and Table I demonstrate the specific localization of antimyosin (Fab′)2 fragments in endocardial and epicardial layers of in-
Infarcted myocardium in four dogs receiving both $^{125}$I-antimyosin (Fab')$_2$ fragments and $^{131}$I-normal IgG (Fab')$_2$ fragments. Nonspecific trapping of IgG (Fab')$_2$ was 38% of the total relative antimyosin (Fab')$_2$ uptake in the center of the infarct zone. Thus, these data indicate that localization of antimyosin (Fab')$_2$ in infarcted myocardium primarily reflects specific antigen-antibody interaction.

Relationship between regional blood flow and antimyosin (Fab')$_2$ uptake in myocardial infarcts. Relative antimyosin (Fab')$_2$ uptake is represented as a function of regional myocardial blood flow, as determined by distribution of $^{85}$Sr-microspheres, in Table II. Relative antimyosin (Fab')$_2$ concentration in dogs after 24 h of coronary occlusion was 5.0±0.5 in myocardial tissue samples where blood flow was reduced to 0–10% of normal flow. In samples where flow was 11–30% of normal, the relative antimyosin (Fab')$_2$ concentration was decreased to 4.0±0.6, and in samples where flow was 31–50% of normal, the relative antibody concentration was only 3.0±0.3. These results demonstrate that antimyosin (Fab')$_2$ uptake was greatest in regions where flow was most severely reduced.

Fig. 7 shows the relationship between regional blood flow and antimyosin (Fab')$_2$ uptake in four dogs undergoing coronary occlusion for 72 h. An inverse exponential relationship between antimyosin (Fab')$_2$ localization and flow was observed. Specific binding of antimyosin (Fab')$_2$ was reduced by reducing flow by 10%, and specific binding was further reduced by reducing flow by 90%.

### Table I

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<th>Location</th>
<th>$^{125}$I-Antibody (Fab')$_2$</th>
<th>$^{131}$I-Normal Ig (Fab')$_2$</th>
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<td>Posterior left ventricle (normal)</td>
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<td>869±16</td>
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<td></td>
<td>Endo 323±8</td>
<td>904±26</td>
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<td></td>
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<td>3,439±91</td>
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<tr>
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<tr>
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### Table II

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<th>51–80</th>
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<td>[%N]</td>
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<td>4.0±0.6</td>
<td>3.0±0.3</td>
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calization and regional blood flow in the experimentally infarcted myocardium is evident ($r = 0.85$, $P < 0.001$). In tissue samples where flow was reduced to 0–10% of normal flow, mean relative antimyosin (Fab')$_2$ uptake was 15.9±0.8. In areas where flow was reduced to 11–30% of normal flow, this ratio was 10.6±0.7. The highest antimyosin (Fab')$_2$ uptake was observed in regions where flow was markedly reduced to 1–2% of nonischemic myocardial blood flow.

**Antimyosin antibody uptake in an uninstrumented animal and in the thoracotomy incision.** To determine whether intrinsic differences in antibody uptake occurred between the anterior and posterior walls of the left ventricle, $^{131}$I-labeled antimyosin antibody was injected intravenously in a normal dog. 24 h later, the animal was sacrificed and multiple myocardial samples taken. The ratio between anterior and posterior left ventricular counts was 1.0±0.1. To determine antibody uptake in the area of a thoracotomy incision, samples of skeletal muscle from this region were taken 24 h after the injection of labeled antimyosin antibody. The ratio of uptake in this region to normal posterior left ventricular myocardium was 1.7±0.2.

**DISCUSSION**

The present studies were undertaken to test the hypothesis that macromolecules could enter, as well as exit from, myocardial cells after ischemic damage to membrane permeability, and that macromolecules with appropriate antibody specificity might therefore localize selectively in these damaged areas. Cardiac myosin was chosen as the intracellular component to be studied with regard to specific antibody binding in vivo for the following reasons: (a) it is the major intracellular protein of the cardiac muscle cell (15); (b) the high molecular weight (≈500,000) (16) and unique solubility properties of intact myosin suggest that it might remain far longer than other intracellular components in the damaged myocardium; this is supported by persistence of extractable myosin in near-normal concentrations from infarcted tissues up to 30 days after the onset of myocardial infarction (17); and (c) unique structural and antigenic features of cardiac myosin have been demonstrated that are not shared by skeletal and smooth muscle myosin (18).

Utilization of antibodies to localize antigenic determinants has been well established for in vitro studies. Fluorescein-labeled antibodies have been used to detect or localize cell membrane antigens (19) and cell membrane-bound immunoglobulins (20), as well as to delineate intracellular structural components in vitro (21).

The present investigation has demonstrated that antibodies specific for cardiac myosin localized in infarcted myocardium. Specific localization was enhanced by using antimyosin (Fab')$_2$ fragments that are two thirds the molecular weight of intact antibodies, but retain bivalency of antibody combining sites (22). The use of (Fab')$_2$ fragments also eliminated the involvement of complement-mediated reactions (23). The data presented are consistent with localization of antibody specific for cardiac myosin (intact or (Fab')$_2$) after the inward diffusion of extracellular macromolecules across membranes altered by ischemic myocardial damage. The presence of intracellular antigen presumably acts as a sink for progressive accumulation of antibody. The specific localization of antimyosin (Fab')$_2$ in relation to the nonspecific trapping of normal IgG (Fab')$_2$ substantiates a specific trapping mechanism based on antigen-antibody interaction. It has been established that after acute myocardial infarction, an increase in permeability of the myocardial cell membrane allows the egress into the plasma of intracellular macromolecular constituents such as creatine phosphokinase (1), serum glutamate oxalacetate transaminase (2), and lactate dehydrogenase (3). The intracellular entry and accumulation of colloidal lanthanum (particle size of 40 Å) after acute experimental ischemia in dogs also reflects ischemia-induced alteration in membrane permeability (24). Our data establish that after acute myocardial infarction, there is also an inward diffusion of extracellular macromolecules across myocardial cell membranes altered by acute myocardial damage.

The inward diffusion of other macromolecules such as iodine-131-labeled bovine serum albumin ($^{131}$I-BSA)
into damaged myocardium was also observed in two dogs (data not shown). Relative uptake of $^{125}$I-BSA in the infarcted myocardium was similar to nonspecific localization of $^{125}$I-normal IgG (Fab')$_2$, but much less than the uptake of cardiac myosin-specific antibody (Fab')$_2$ fragments.

Localization of antimyosin (Fab')$_2$ fragments in infarcted myocardium was also observed to be inversely related to regional blood flow as determined by distribution of $^{85}$Sr-microspheres. In ischemic myocardial regions where flow was minimally decreased, uptake of antimyosin (Fab')$_2$ was only slightly above background. With diminishing flow, relative antimyosin (Fab')$_2$, uptake was observed to increase exponentially. At approximately 2% of nonischemic myocardial blood flow, maximal antimyosin (Fab')$_2$ localization was obtained. With the quantity of microspheres used in these studies, no significant alterations in hemodynamics or in regional blood flow to myocardium have been observed (25). Studies employing the microsphere technique to determine regional blood flow in ischemic myocardium in dogs have also shown the reduction of regional flow to be proportional to the severity of myocardial damage (26). Thus, it can be concluded that antimyosin (Fab')$_2$ localization is greatest in regions where flow was most severely reduced, and where myocardial damage was most extensive.

The data presented in this investigation support the hypothesis that ischemia-induced alteration in myocardial cell membrane permeability that allows the efflux of intracellular macromolecules also permits the influx of extracellular macromolecules to the intracellular space. Antibody specific for cardiac myosin localized selectively and specifically in infarcted myocardium in this in vivo canine experimental model, and the concentration of antimyosin (Fab')$_2$ fragments increased with increasing severity of myocardial damage and showed an inverse exponential relationship to regional blood flow in infarcted myocardium. These properties suggest that labeled specific antibodies or fragments of antibodies may represent a conceptually new imaging approach to myocardial infarct localization and sizing.

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