Early Membrane Damage During Coronary Reperfusion in Dogs

DETECTION BY RADIOLABELED ANTICARDIAC MYOSIN $(FAB')_2$

LAWRENCE H. FRAME, J. ANTONIO LOPEZ, BAN AN KHAW, JOHN T. FALLON, EDGAR HABER, and WM. JOHN POWELL, JR., *Cardiac Unit, Massachusetts General Hospital and the Departments of Medicine and Pathology of the Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114*

ABSTRACT There is currently great interest in acute coronary reperfusion as a therapeutic modality for severe myocardial ischemia. While some studies have demonstrated a reduction in the overall extent of necrosis by early reperfusion, other studies have identified potentially deleterious effects produced by reflow. Because membrane disruption may be an important mechanism of irreversible cell injury, we measured changes in cell membrane integrity early during reperfusion using radiolabeled anticardiac myosin $(Fab')_2$ antibody fragments in dogs. Our method involved brief periods of exposure to the $(Fab')_2$ so that the levels of $(Fab')_2$ binding indicated the degree of membrane disruption at discrete times during the progression of cell injury. In the first protocol $(Fab')_2$ fragments labeled with either ^{125}I and ^{131}I were injected into the left circumflex coronary artery at the onset of reflow and at 45 min of reflow after a 1-h circumflex artery occlusion. Coronary sinus flow was diverted for 5 min following each injection to prevent recirculation. The $(Fab')_2$ binding ratio (ischemic/control) increased during the first 45 min of reflow in each of eight experiments (mean increase 170%, $P < 0.01$). No significant increase in $(Fab')_2$ binding was observed in five additional experiments in which nonspecific $(Fab')_2$ was injected. This indicates that the increase in binding seen with antimyosin-specific $(Fab')_2$ was due to changes in specific binding rather than to alterations in $(Fab')_2$ delivery produced by changes in blood flow distribution. The increase in membrane damage during reflow was confirmed by a second protocol in which each animal received only a single left atrial injection of $(Fab')_2$ followed by rapid excision of the heart. The $(Fab')_2$ binding ratio was 1.7±0.3 (SEM) in the group that received $(Fab')_2$ at the onset of reflow and 3.7±0.6 (SEM) ($P < 0.05$) in the group that received $(Fab')_2$ after 45 min of reflow. In a third set of experiments in which hyperosmotic mannitol was infused during reflow the mean increase in $(Fab')_2$ binding using the first protocol was only 80±40 vs. 170±30% without mannitol ($P < 0.05$). Thus, membrane damage develops early during coronary reperfusion following 1 h of circumflex coronary artery occlusion, and part of this membrane damage can be prevented by altering the conditions of reflow. A method involving brief exposure of the myocardium to antimyosin $(Fab')_2$ is promising for detecting changes in membrane integrity during evolving ischemic injury.

INTRODUCTION

Reperfusion is now an important modality for treating myocardial ischemia produced by coronary occlusion. Both mechanical (1–3) and pharmacologic (4–8) methods for restoring perfusion are being used with increasing frequency in an attempt to prevent or limit cell necrosis. Reflow provides the metabolic substrates for the process of recovery from reversible ischemic injury. However, during the period of recovery cells may be vulnerable to stresses produced by reflow. Several studies have identified events occurring during reflow that may contribute to cell death (9–16). In a previous report, we showed under certain circumstances that cells which are potentially salvageable at the end of the period of occlusion undergo irreversible

Address reprint requests to Dr. W. J. Powell, Jr., Cardiac Unit, Massachusetts General Hospital, Boston, MA 02114.

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cell damage during reperfusion (14). Jennings (17) has stated that the development of defects in the sarcolemma may be the primary event causing the transition from reversible to irreversible cell injury in ischemia.

Radiolabeled antibodies to cardiac myosin and their $(Fab')_2$ fragments, which were developed as a specific method of identifying myocardial necrosis (18–23), have been shown by electron microscopy to be localized at sites of cell membrane disruption (24). Therefore, in this study, we have investigated changes in cell membrane integrity during reflow following coronary occlusion in a canine model using anticardiac myosin $(Fab')_2$. Furthermore, we have determined whether at least some of the membrane damage is produced by reflow or is an inevitable consequence of the period of coronary occlusion.

This study involved a new application of antimyosin antibodies as a marker for loss of cell membrane integrity early during the course of ischemic injury. Antimyosin $(Fab')_2$ has several advantages as a marker of cell membrane damage. It may be delivered in vivo in tracer amounts without affecting the course of the experiment. In addition, because of high specificity and affinities of antibodies, its activity can be localized and quantitated at the end of the experiment by tissue counting. The method of delivering $(Fab')_2$ developed for this study allowed sequential measurements of membrane damage at specific times during the evolution of ischemic injury during reflow.

METHODS

Preparation of anticardiac myosin $(Fab')_2$. The purification of canine cardiac myosin, immunization of rabbits, purification of rabbit antimyosin antibody using a myosin-Sepharose immunoabsorbent have been described previously (18). Anticardiac myosin $(Fab')_2$ antibody fragments were obtained from the intact antibody by pepsin digestion at 37°C for 20 h at pH 4.5 using the method of Edelman (25) and Marchalonis (26). Antimyosin $(Fab')_2$ was iodinated with ^{125}I or ^{131}I using the lactoperoxidase procedure of Marchalonis (26). Before injection, the antimyosin $(Fab')_2$ was passed through a millipore filter with a pore size of 0.22 μm (Millipore Continental Water Systems, Bedford, MA) coated with bovine serum albumin.

Experimental ischemia. Experiments were performed in 30 adult mongrel dogs weighing between 20 and 25 kg, which were anesthetized with intravenous pentobarbital (30 mg/kg). The animals were ventilated with 97% O_2–3% CO_2 after endotracheal intubation. Each dog received procainamide (Pronestyl) 1,000 mg i.m. divided into three sites and heparin 6,000 U i.v. Arterial and venous pressures were monitored from cannulae placed in a femoral artery and vein using Statham P23Db transducers (Statham Instruments, Inc., Oxnard, CA). The chest was opened using a left thoracotomy and the heart was suspended in a pericardial cradle.

In each experiment a 1-h coronary occlusion was produced by tightening a ligature around the circumflex coronary artery within 1.5–2 cm from its origin and before any major epicardial marginal branches. When premature ventricular contractions developed after coronary occlusions or during reflow they were treated with bolus intravenous injections of xylocaine 25 mg. 24 of 27 dogs subject to ischemia received an average of 85±11 (SEM) mg of lidocaine. The amount of lidocaine delivered did not correlate with the magnitude of $(Fab')_2$ binding. An additional four dogs developed ventricular fibrillation during occlusion; they were not resuscitated and were not included in these data. Two separate protocols were used, which differed in the length of reflow, the number and site of $(Fab')_2$ injections, and the method for limiting recirculation of $(Fab')_2$.

In the first protocol two injections of 300 μCi of antimyosin $(Fab')_2$ were given to each animal and reflow was continued for 6 h after release of the coronary occlusion. The $(Fab')_2$ was injected directly into the left circumflex artery through a small nonocclusive plastic cannula placed just distal to the coronary ligature (Fig. 1). The first injection was given immediately prior to release of the occlusion at the onset of reflow. A second injection of $(Fab')_2$ with a different radiolabel was given after 45 min of reflow. ^{125}I-Labeled $(Fab')_2$ was injected first in four experiments and ^{131}I-labeled $(Fab')_2$ was injected first in the other four.

In these 6-h reflow experiments total coronary sinus flow was diverted and discarded during and for 5 min after the $(Fab')_2$ injection to limit recirculation of the antibody fragments. Coronary sinus diversion was established as follows. A cannula was introduced through the right atrial appendage into the ostium of the coronary sinus (Fig. 1). A suture was passed through the right atrial wall and completely encircling the coronary sinus. When the suture was tied tightly all coronary sinus flow passed through the coronary sinus cannula. This occlusive ligature was tested by demonstrating that blood continued to be pumped through the cannula when the open external end was held above right atrial pressure. The coronary sinus cannula was connected to a Y connector. One branch returned blood to the right atrium and the other allowed external drainage of the coronary sinus blood. When the drainage cannula was clamped (solid arrow, Fig. 1) the blood was recycled into the right atrium. During periods of diversion of coronary sinus blood the right atrial return cannula was clamped (open arrow, Fig. 1) and the external drainage cannula was released. The blood from one donor dog anesthetized with methohexital (Brevital, Eli Lilly & Co., Indianapolis, IN) (25 ml of 1% solution in saline) was infused intravenously as needed to replace the 200–400 ml of coronary venous effluent that was drained during the 5-min coronary sinus diversion.

The effectiveness of the coronary sinus diversion was evaluated in two preliminary experiments in which frequent blood samples from the coronary sinus drainage and the venous blood were collected for gamma counting after a single intracoronary injection of $(Fab')_2$. Data from one of these experiments are shown in Fig. 2. During the experiments that used two serial intracoronary injections of $(Fab')_2$ the ratio of peak coronary sinus activity to peak venous activity averaged 184±41 SEM. Thus, the concentration of $(Fab')_2$ is much greater in the first pass after intracoronary injection, as reflected by peak coronary sinus activity, than it was during recirculation, as reflected by venous activity. The peak activity in the circumflex artery distribution must in fact exceed the measured coronary sinus activity, which is diluted by nonradioactive blood from other areas of the heart. Furthermore, the concentration of recirculation $(Fab')_2$ falls rapidly during the first hour from the peak level in venous blood.

The circumflex artery catheter used for injecting the

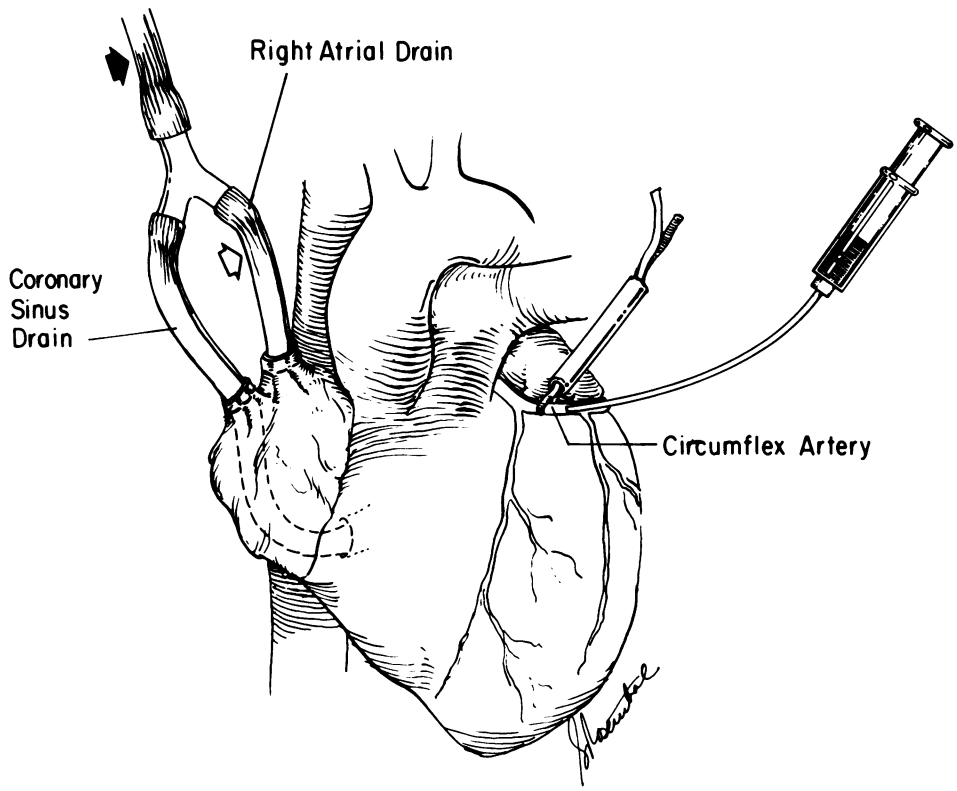

FIGURE 1 Instrumentation for experiments involving serial intracoronary injections of anti-myosin $(Fab')_2$ with coronary sinus diversion to limit recirculation (see text for details).

$(Fab')_2$ was removed after the second injection. To determine whether the presence or the removal of the circumflex catheter adversely affected coronary perfusion, an experiment was performed in which coronary perfusion pressure in a circumflex marginal branch was measured. Coronary perfusion pressure distal to the catheter site was not lowered >0–3 mmHg, compared with aortic pressure either by the presence of the coronary catheter or by bleeding after it was removed. Thus, neither the coronary catheter nor its removal significantly affected perfusion of the area of the myocardium supplied by the circumflex coronary artery.

Five other experiments also used the first protocol except that two injections of nonspecific $(Fab')_2$ fragments were injected instead of anticardiac myosin-specific $(Fab')_2$. These experiments were performed to evaluate whether changes in $(Fab')_2$ were due to changes in specific binding of $(Fab')_2$ because of changes in membrane integrity or were artifacts of alterations of blood flow in the ischemic area during reflow.

In seven additional experiments the protocol was identical except that a 20% mannitol solution was infused into the left atrium through a cannula in the left atrial appendage. Mannitol was infused beginning 5 min before release of the occlusion at a rate of 38.2 ml/min for 5 min, 15.2 ml/min for the next 10 min, and 7.64 ml/min for the next 15 min. This procedure produced a rapid increase in serum osmolality of 36±6 (SEM) mosmol in the first 5 min followed by a gradual increase to 45±6 (SEM) mosmol above control following 30 min of infusion.

Three control experiments with long reflow were performed to study the distribution of $(Fab')_2$ injected into the circumflex coronary when no infarction was present. ^{125}I-Labeled $(Fab')_2$ was injected into the circumflex coronary during a 10-s coronary occlusion and then reflow was continued for 6 h. The coronary sinus diversion was used to limit recirculation of the antibody fragments.

The second protocol differed from the first in that ^{125}I-$(Fab')_2$ was injected into the left atrium to avoid the selective distribution to the circumflex area. In these experiments coronary sinus diversion would not limit recirculation and was not used. Therefore, the heart was quickly excised 15 s after the $(Fab')_2$ was injected either at the onset of reflow or after 45 min of reflow. This period of exposure before excising the heart was chosen after measuring the time from aortic arch injection of fluorescein to its first appearance in the myocardium in the first two experiments. In both cases this circulation time was 20 s. Thus, excising the heart prevented recirculation after the first pass of $(Fab')_2$ through the myocardium. Because of a higher level of circulating ^{125}I-$(Fab')_2$ in the blood in these experiments the aortic root was cannulated after the heart was excised and coronaries were perfused with 1 liter of lactated Ringer solution to reduce the background radioactivity contained within the vascular space and not bound to damaged cells. In seven of these experiments (three with 15 s of reflow, four with 45 min and 15 s of reflow) 9 μCi of ^{141}Ce-microspheres were injected into the left atrium immediately before the injection of ^{125}I-antimyosin $(Fab')_2$. There was a lack of correlation between

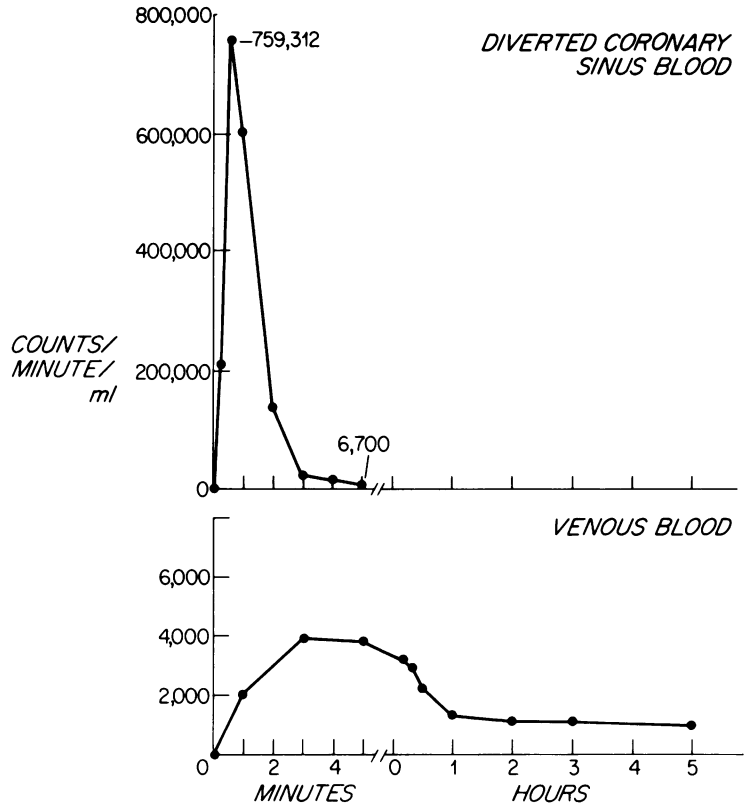

FIGURE 2 Radioactivity in diverted coronary sinus blood (*above*) and recirculating venous blood (*below*) as a function of time following a single injection of ^{125}I-labeled anticardiac myosin $(Fab')_2$ into the left circumflex coronary artery. The time scale is expanded for the first 5 min, which corresponds to the period of coronary sinus diversion. Note that at the end of 5 min the coronary sinus radioactivity has fallen to <1% of the peak level. Furthermore, though some $(Fab')_2$ escaped the coronary sinus diversion because a small fraction of the coronary venous blood drains directly into the left ventricle, the amount that continues to recirculate falls rapidly during the first hour and is much less than the concentration in the first pass through the heart as reflected by the peak coronary sinus level.

the ratio of $(Fab')_2$ in the ischemic tip of the posterior papillary muscle to a similar ratio for blood flow determined by microspheres. (r value of 0.35, not statistically significant [$P < 0.45$]).

Tissue analysis. At the end of each experiment the heart was perfused with triphenyl tetrazolium chloride (TTC)[1] 250 ml to aid in the identification of the infarcted area and the selection of tissue samples (28, 29). This was performed in vivo by intravenous infusion over 20 min in the long reflow experiments and by aortic perfusion in the rapid excision experiments. Tissue samples were taken from a longitudinal section of the left ventricle that included the full length of both papillary muscles. Experimental samples were taken from the distal third (area 1) and middle third (area 2) of the posterior papillary muscle, the basal (area 3) and apical (area 4) subendocardial muscle, and basal (area 5) and apical (area 6) subepicardial muscle. All of these samples came from muscle in the circumflex coronary artery distribution. Most of our results focus on the sample from the distal one-third of the papillary muscle because previous studies (10–12, 27) show severe and reproducible ischemic injury and very little if any collateral flow to this area during circumflex artery occlusion. In this study we found a consistent lack of staining by TTC in this region indicating severe ischemic injury. The other samples in the ischemic area are less severely injured than the tip of the papillary muscle in this model (27). In this study areas 2, 3, and 4 also showed lack of TTC staining, although the pattern was more patchy than area 1. Areas 5 and 6 showed only a slight difference in TTC staining from normal, indicating less severe ischemic injury. In addition, a total of five control samples were taken from the nonischemic anterior papillary muscle, and subendocardial and subepicardial portions of the high and low anterior wall adjacent to the anterior papillary muscle.

The sections were counted in a gamma counter and radioactivity was expressed per gram of wet fixed tissue. There was no systematic difference between endocardial and epicardial activity. In most hearts the anterior papillary muscle

[1] *Abbreviation used in this paper:* TTC, triphenyl tetrazolium chloride.

was not infarcted as detected by TTC and the counts per gram did not differ from samples taken from the anterior wall. In two hearts the TTC stain showed evidence of ischemic damage to a portion of the interior papillary muscle and the specific radioactivity was greater than in the samples from the anterior wall. In these hearts the anterior papillary muscle sample was not averaged with those from the anterior wall in obtaining the averaged noninfarcted control activity.

The level of specific binding of antimyosin $(Fab')_2$ was indicated by the $(Fab')_2$ binding ratio, which is defined as the ratio of counts per gram in each ischemic muscle sample to the average counts per gram in the nonischemic control areas.

The statistical significance of differences between nonpaired groups of data was evaluated using Kruskal-Wallis test followed by the Mann Whitney test for differences between groups (30). A Wilcoxon signed rank test was used for paired data. These nonparametric tests were used because data points were not normally distributed (30). Variation from mean values were given as the standard error of the mean.

RESULTS

In eight animals antimyosin $(Fab')_2$ with two different radiolabels was injected into the circumflex coronary artery at the onset of reflow and after 45 min of reflow, respectively. The coronary sinus diversion system was used to limit recirculation. The paired data of the $(Fab')_2$ binding ratio for the tip of the posterior papillary muscle (area 1) from these experiments is shown in Fig. 3 *A*. It can be seen that the binding of antimyosin $(Fab')_2$ after 45 min of reflow exceeds the binding at the onset of reflow in every experiment. This change is statistically significant using a nonparametric Wilcoxon sign rank test for paired data with a $P < 0.01$. Because of differences in the absolute magnitude of the $(Fab')_2$ binding ratios between dogs, the magnitude of the change can be more easily seen when the data are normalized as in Fig. 3 *B*. This figure shows the percent increase in $(Fab')_2$ ratio during the first 45 min of reflow for each of the eight experiments. The mean increase was 170±35% (SEM) with a range of increases from 80 to 320%.

The mean increase in the $(Fab')_2$ binding ratio for the middle third of the posterior papillary muscle (area 2) and the basal and apical subendocardial tissue was 136±36, 116±32, and 106±26%, respectively. These increases in $(Fab')_2$ binding were also significant, although the magnitude of the increase is less on average than in the more consistently and severely injured tissue from area 1. In contrast, the increase in $(Fab')_2$ binding ratio in areas 5 and 6 from the subepicardial portion of the ischemic zone were only 8±30 and 18±22%, which were not significant.

Fig. 3 *C* shows the percent change in $(Fab')_2$ binding in the tip of the papillary muscles in five experiments using the same protocol except that nonspecific $(Fab')_2$ was injected instead of antimyosin $(Fab')_2$. In these experiments there was only a 26±22% increase in nonspecific $(Fab')_2$ binding during the first 45 min of reflow and this change was not statistically significant. These experiments indicate that the increased binding of antimyosin $(Fab')_2$ during reflow is due to a true increase in exposed myosin through sites of membrane disruption rather than alterations of blood flow in the ischemic area affecting delivery or washout of the $(Fab')_2$ fragments.

In three control experiments the circumflex coronary artery was not occluded. A single injection of radiolabeled $(Fab')_2$ was given into the circumflex artery during coronary sinus diversion and the experiment was continued for 6 h after injection just as in the occlusion-reflow experiments. These experiments were performed to determine the extent to which $(Fab')_2$ binding in the posterior papillary muscle is enhanced over binding in areas in the distribution of the left anterior descending coronary artery because of selective injection into the circumflex coronary artery per se. The binding ratios in area 1 for these three experiments were 1.3, 1.3, 2.2 (mean 1.6) compared with an absolute mean binding ratio of 5.3±1.8 after an hour of circumflex artery occlusion and 16.8±8.1 after the hour of occlusion followed by 45 min of reflow.

Another set of experiments was performed to exclude any artifacts due to unequal concentrations of isotope in the normal and ischemic areas or the effects of delayed binding in the ischemic area due to the small amounts of recirculating labeled $(Fab')_2$. A single injection of $(Fab')_2$ was given into the left atrium to allow thorough mixing before the blood reached the coronaries. $(Fab')_2$ was injected either immediately after release of the coronary occlusion (group I, $n = 6$) or after 45 min of reflow (group II, $n = 6$). In each case the heart was rapidly excised 15 s after the antibody injection so only the first pass of the radioactive $(Fab')_2$ entered the coronaries. The mean $(Fab')_2$ binding ratio at 0 min of reflow was 1.7±0.3 (SEM). The $(Fab')_2$ binding ratio reflecting membrane damage after 45 min of reflow was significantly increased to 3.7±0.6 ($P < 0.05$).

The preceding experiments established that an increase in membrane damage indicated by antimyosin $(Fab')_2$ binding occurs during the first 45 min of reflow. We next wished to address the question of whether this membrane damage occurs as a result of detrimental effects of reflow. We studied the influence of elevating serum osmolality by mannitol just before the time of reflow on the increase in binding of $(Fab')_2$ during reflow. The administration of hyperosmotic mannitol significantly attenuated the increase in $(Fab')_2$ binding during the first 45 min of reflow from 170±30

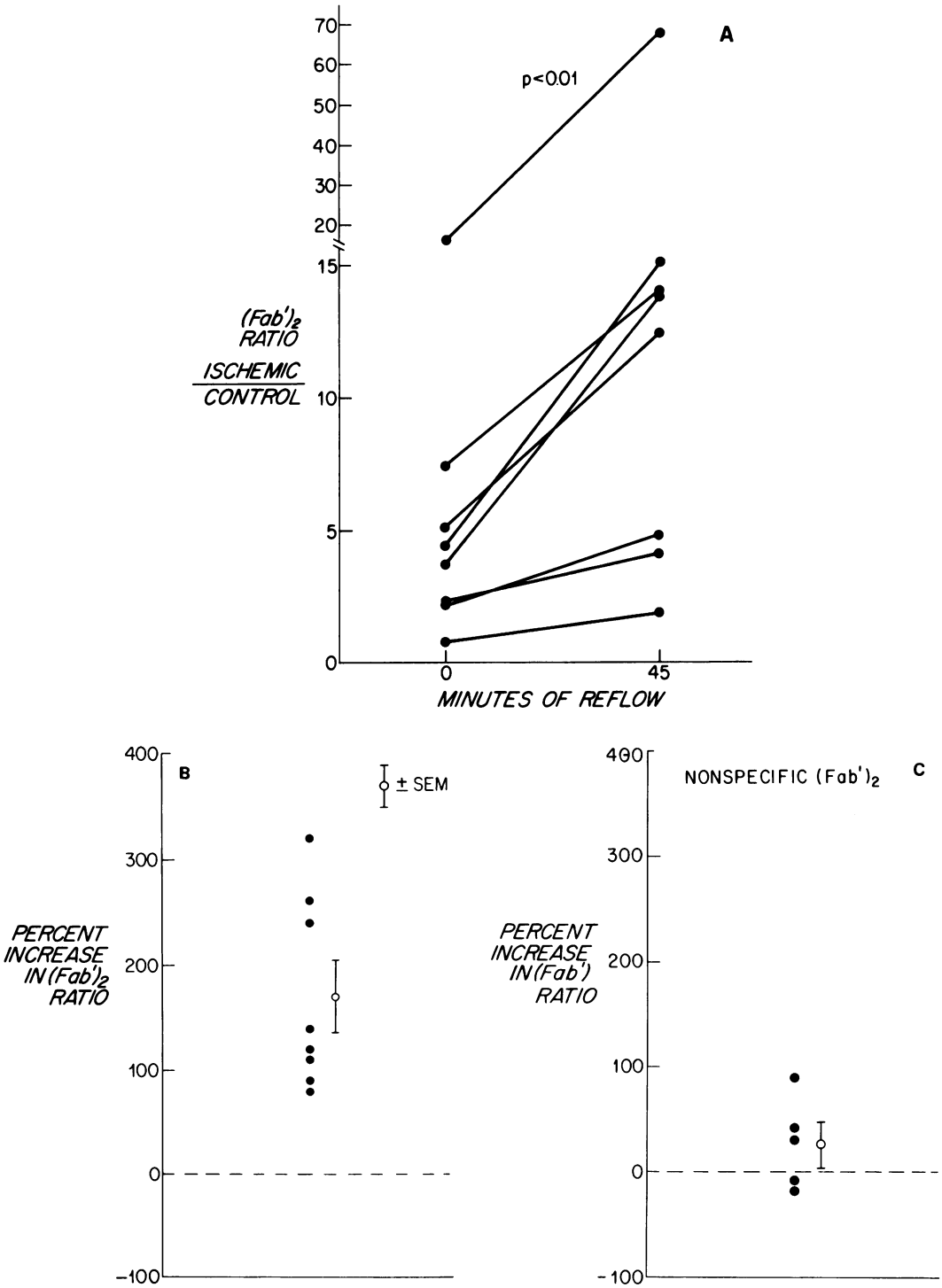

FIGURE 3 (*A*) Paired data from eight experiments with serial intracoronary injections of $(Fab')_2$ showing the change in membrane damage in the ischemic tip of the posterior papillary muscle during the first 45 min of reflow as indicated by the $(Fab')_2$ binding ratio. The $(Fab')_2$ binding ratio is the radioactivity (counts per gram) in the ischemic area divided by the activity in the control areas. Note that $(Fab')_2$ binding increased during reflow in every experiment. (*B*) Percent increase in the $(Fab')_2$ binding ratio at 45 min of reflow compared with the ratio at the beginning of reflow for the same eight experiments shown in panel *A*. (*C*) Percent increase in $(Fab')_2$ binding ratio at 45 min of reflow compared with the ratio at the beginning of reflow for five experiments in which nonspecific $(Fab')_2$ was used instead of anticardiac myosin $(Fab')_2$.

(SEM) to 80±40%; $P < 0.05$ using the Wilcoxon rank sum test.

DISCUSSION

These data indicate that loss of cell membrane integrity occurs very early during reflow following coronary occlusion. At least part of this membrane damage is produced by the acute reperfusion. The study also demonstrates the feasibility of sequential measurements of membrane damage during evolving ischemic injury using antimyosin $(Fab')_2$.

The conclusion that membrane damage results from events that occur early during reperfusion is based on the ability of a pharmacologic intervention during reperfusion to reduce the membrane damage. If loss of membrane integrity during reflow occurred only as an inevitable delayed consequence of ischemic injury during the period of occlusion it would not be altered by modifying the conditions of reflow. These results focus attention on the impact of acute blood reflow on the early progression of cell injury following ischemia.

This study involved a new application of specific antibody fragments as a cellular probe. We detected membrane damage at specific times by exposing the heart to antimyosin $(Fab')_2$ only for brief periods. In one protocol we detected changes in membrane integrity using sequential bolus injections of anticardiac myosin $(Fab')_2$ fragments. This method has the advantage of providing paired comparisons from each experimental animal. Limiting recirculation of the $(Fab')_2$ was an essential component of this method in order that the $(Fab')_2$ binding would reflect the amount and distribution of membrane damage present at the time of injection and not membrane damage that develops later. Significant recirculation of $(Fab')_2$ would have reduced the sensitivity of the technique by blunting the difference in binding between injections at different times. Coronary sinus diversion after intracoronary $(Fab')_2$ injection appeared to limit recirculation adequately, because we found consistently greater binding from the second of two injections. The increase in $(Fab')_2$ binding detected by serial injections into the coronary artery were confirmed by experiments that used a single intraatrial injection of $(Fab')_2$. With this protocol, both ischemic and normal areas received the same concentration of $(Fab')_2$, and recirculation was completely prevented by rapid excision of the heart. Comparing the data from these two protocols the increases in $(Fab')_2$ binding ratio during reflow were similar, which supports the validity of serial injections with limited recirculation for detecting changes in membrane damage during evolving ischemic injury.

Our primary focus of investigation was on the distal third of the posterior papillary muscle because it has been extensively studied and shows severe and consistent injury in this model (10–17, 27). We also examined changes in $(Fab')_2$ binding in other regions with a spectrum of ischemic injury in the eight experiments using the first protocol. $(Fab')_2$ binding increased significantly in tissue from the base of the papillary muscles and subendocardial muscle in the ischemic area. These areas also showed severe ischemic changes after comparable periods of circumflex occlusion followed by reflow, although to a lesser degree than the tip of the papillary muscle (27). We did not find an increase in $(Fab')_2$ binding during reflow in subepicardial tissue taken from the ischemic area supplied by the circumflex coronary following 1 h of coronary occlusion. Reimer et al. (27) has shown that only minimal necrosis is present in this region even after a 3-h occlusion followed by reflow whereas after a 24-h permanent occlusion necrosis is present in this area. In light of these data it appears that the progression of membrane disruption during reflow occurs in severely ischemic areas but that less severely ischemic regions that can clearly be salvaged by reflow alone may not develop this membrane damage.

Antimyosin antibody binding has been shown to indicate cell membrane disruption associated with irreversible cell injury. Khaw et al. (24) using scanning electron microscopy have demonstrated that antimyosin antibody is a marker of membrane damage. They have shown specific binding of anticardiac myosin at localized sites of membrane disruption. They also showed that the amount of $(Fab')_2$ bound to single cells in tissue culture provides a quantitative method of sorting viable from irreversibly injured cells. Cultured murine myocytes were injured by exposure to glucose-free medium and then separated according to the number of antimyosin-fluorescent spheres on the cell surface. Cells that demonstrated enhanced binding were nonviable, whereas cells without increased binding continued to contract and grow in culture. Khaw et al. (21) have shown that antimyosin $(Fab')_2$ binding in vivo specifically identified necrotic cells between 4 and 48 h after coronary occlusion. $(Fab')_2$ binding was localized to cells with histologic evidence of necrosis and was absent from surviving cells in the infarcted area. In models of infarction involving both permanent occlusion and occlusion-reflow, when antimyosin $(Fab')_2$ has been given intravenously several hours after coronary occlusion in dogs, there has been a direct quantitative relation of the severity of ischemia in different areas indicated by reduction of blood flow using microspheres and binding of specific $(Fab')_2$ (18, 19, 23).

In the present study, therefore, there is justification for using changes in antimyosin $(Fab')_2$ binding as an

indicator of changes in cell membrane integrity as long as other factors that may alter its distribution are not present. It is important to consider whether the increase in $(Fab')_2$ binding we observed during reflow might be due to alterations of blood flow affecting delivery or washout of the antibody fragments rather than a true increase in specific binding sites. Impaired flow during the injection could diminish $(Fab')_2$ binding by decreasing its delivery to ischemic areas. On the other hand, normal delivery followed by impaired or absent washout could elevate the apparent $(Fab')_2$ binding. Therefore, impaired blood flow could either increase or decrease apparent $(Fab')_2$ binding. We did not find a significant correlation between changes in blood flow and $(Fab')_2$ binding in seven experiments in which radiolabeled microspheres were injected at the time of $(Fab')_2$ injection. However, such blood flow data are not sufficient, by themselves, to exclude artifacts due to factors that alter delivery or washout of $(Fab')_2$ for several reasons. First of all the blood flow at the time of injection does not necessarily reflect the blood flow during the period of washout, which in our first protocol lasts 6 h. Secondly, to establish with a high degree of confidence that there was no correlation might require a very large number of experiments. Third, the lack of a correlation between average blood flow and average $(Fab')_2$ binding within tissue samples does not exclude a significant influence of blood flow at the microvascular level because there may be heterogeneity with respect to impaired reflow and/or washout within each tissue sample. Fourth, even a positive correlation between $(Fab')_2$ binding the blood flow during reflow at the time of $(Fab')_2$ injection does not prove that the amount of blood flow was responsible for the level of $(Fab')_2$ binding. In fact, under the conditions of our experiments areas with normal, excess, or moderately diminished flow would be exposed to the same high intravascular concentration for the same period of time. Since the intravascular concentration of $(Fab')_2$ was very high, the amount of antibody leaving the capillary lumena into the tissue might not be flow limited until flow was severely reduced or totally eliminated.

We evaluated the question of whether the increase in $(Fab')_2$ binding during reflow could have been an artifact of altered delivery or washout of the $(Fab')_2$ fragments at either of the two times during reflow using nonspecific $(Fab')_2$ fragments instead of antimyosin $(Fab')_2$ in the first protocol. Since the distribution of nonspecific $(Fab')_2$ would be influenced by variations in delivery to the tissue, capillary permeability, and washout, to the same extent as antimyosin-specific $(Fab')_2$, the difference between these groups of experiments is due to differences in specific binding of antimyosin $(Fab')_2$. The binding of nonspecific $(Fab')_2$ did not increase during the first 45 min of reflow. Therefore, the increase in antimyosin $(Fab')_2$ binding during reflow is due to an increase in available binding sites on myosin protruding through sites of membrane disruption.

Previous data have shown that hyperosmotic mannitol infusion during reflow reduces the degree of eventual necrosis detected histologically (9, 14, 15). We have therefore used antimyosin $(Fab')_2$ in this study to detect the effect of mannitol infusion on early changes in membrane integrity. The ability to improve cell salvage by an intervention during reflow suggests that many cells, which eventually became necrotic, are not yet irreversibly injured at the onset of reflow. The exact mechanism by which reflow may contribute to irreversible injury of ischemic cells is not known, but several investigators have suggested that cell swelling may be important (8, 13, 16, 17). Jennings (17) has stated that loss of cell volume regulation is an early change in ischemic injury. Explosive cell swelling is known to occur early during reflow following myocardial ischemia (13). Powell et al. (14) and DiBona et al. (16) found a correlation between prevention of cell swelling and reduction in eventual necrosis by hyperosmotic mannitol delivered during reflow.

There are at least two mechanisms by which cell swelling may contribute to cell necrosis. Leaf (31) and Powell et al. (14) suggested that cell swelling may lead to necrosis of other cells by producing the no-reflow phenomenon. On the other hand it is conceivable that cell swelling may directly injure cells. Jennings (17) noted that defects in the cell membrane have been difficult to demonstrate after 60 min of ischemia unless "the affected cells have been allowed to swell" either during blood reflow in vivo or tissue incubation in vitro. He also suggested that defects of the sarcolemma may contribute to irreversible cell injury. It is therefore possible that cell swelling directly contributes to irreversible injury by promoting sarcolemmal defects. According to this hypothesis, the ability of mannitol to attenuate the development of membrane disruption in the present study and to reduce eventual necrosis (16) in this model could be directly attributable to the reduction of cell swelling.

Although the present study demonstrates early changes in membrane integrity during reflow, other potentially deleterious effects of reperfusion have been previously identified. Some studies of reperfusion injury have focused on myocardial hemorrhage that may develop several hours after reperfusion (32). However, in the model used in this study, extravasation of erythrocytes is not seen grossly or histologically after the first 6 h of reflow. It therefore appears that there may be at least two distinct forms of injury produced by reflow.

It should be emphasized that even if reflow had deleterious effects on some severely injured cells, we do not mean to imply that the net effect of reflow is necessarily negative. For example, in the present study there was no increase in $(Fab')_2$ binding in subepicardial tissue where ischemia was less severe. In other studies, reflow within the first few hours of experimental coronary occlusion has been shown to decrease the extent of necrosis (27, 33, 34) and to improve survival (35). However, if reperfusion has detrimental effects in some parts of the ischemic zone that can be reduced by optimizing the conditions of reflow, then greater salvage may be possible. The possibility of developing improved methods for coronary reperfusion would be clinically important because of the current interest in the effects of reperfusion in patients by surgical revascularization, thrombolysis, and relief of coronary spasm following acute coronary occlusion. Furthermore, interventions that are beneficial when delivered only during reflow have the practical advantage of not requiring pretreatment or chronic therapy of patients at risk for myocardial infarction.

Further investigation is needed into possible detrimental effects of reflow and evaluation of interventions that may diminish these effects or hasten cell recovery during reflow. The present study provides evidence for increasing sarcolemmal disruption early during reflow, which can be modified by an osmotic intervention. Furthermore it demonstrates the usefulness of our method of sequential injections of anticardiac myosin $(Fab')_2$ as a sensitive indicator of changes in cell membrane integrity in experimental studies of evolving ischemic injury.

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