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Patterns of cellular injury in myocardial ischemia determined by monoclonal antimyosin

(immunofluorescence/heterogeneity/myocardial infarction/border zone)

A. CLARK NOLAN*, WILLIAM A. CLARK, JR.*, THEODORE KARWOSKI*, AND RADOVAN ZAK*†

Departments of *Medicine and †Pharmacological and Physiological Sciences, University of Chicago, Chicago, Illinois 60637

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ABSTRACT **The development of cellular injury in the rat left ventricle resulting from left coronary artery occlusion was examined by immunofluorescence after intravenous injection of monoclonal antimyosin. Cardiac muscle cells that bound antimyosin during ischemia were localized by staining sections with fluorescein-conjugated anti-mouse IgG. Fluorescent staining was detectable within the ischemic region of the left ventricle 3 hr after occlusion and injection of antimyosin. After 6 hr of ischemia, the highly irregular margin of the ischemic zone was clearly outlined by fluorescent cells. At 3–6 hr after occlusion, marked heterogeneity in cellular staining was observed in the epicardial half of the ischemic area, with intensely fluorescent cells intermixed with cells of markedly lower fluorescence. By 24 hr, a homogeneous pattern of staining was observed throughout the ischemic zone. In nonischemic regions of the heart and in rats treated for 24 hr with antimyosin without occlusion, there were only background levels of staining. We conclude that: (*i*) visualization of ischemic cells via antimyosin provides a sensitive means for examining developing patterns of injury; (*ii*) the heterogeneity of staining during early ischemia may reflect variation in cellular resistance to deprivation; and (*iii*) the pattern of fluorescence at the margin of the occluded region indicates that the "border zone" is composed of interdigitating ischemic and nonischemic tissues.**

The temporal and spatial distribution of myocardial injury developing in response to coronary occlusion has been the subject of many investigations. In contrast to emerging techniques such as scintiscans, positron emission tomography, and NMR, which provide assessment of ischemic zones, methods have not been exploited by which damaged cells would be individually labeled and directly visualized microscopically. A suitable method would unequivocally identify injured cells and permit large areas to be surveyed quickly. Such a method requires the development of a probe that penetrates cell membranes and remains confined to the cells. Defects in the plasma membrane of ischemic myocytes were observed by Jennings *et al.*, occasionally after 1 hr, commonly after 2 hr of coronary occlusion, and were considered a manifestation of irreversible injury (1, 2). In work by Shell and colleagues, creatine phosphokinase appeared in the circulation in proportion to its depletion in ischemic myocardium (3). The loss of this enzyme is a manifestation of the increased permeability of ischemic cell membranes. Haber and co-workers demonstrated the preferential uptake of isotopically labeled specific antibodies to myosin in ischemic heart muscle (4–6). Apparently, analogous to the egress of macromolecular enzymes, the antibodies penetrated defective cell membranes to bind to the essentially insoluble intracellular protein, myosin.

In this report, we describe the use of fluorescein-labeled monoclonal anticardiac myosin to examine the patterns of developing ischemic injury at the cellular level. The results of this study provide a high-resolution image of the border zone of ischemic regions as well as visual evidence for the heterogeneous development of cellular injury during early phases of ischemia.

MATERIALS AND METHODS

Sprague–Dawley rats (300–400 g) were anesthetized with ether and given 4–6 mg of lidocaine intraperitoneally to combat arrhythmia. Ventilating the lungs, the left thorax was entered and the heart exposed. The left coronary artery (LCA) was ligated as described by Selye *et al.* (7). When LCA occlusion (LCAO) was complete, antimyosin was injected intravenously. The thorax was then closed and the rats allowed to recover for 3–24 hr.

Monoclonal antibodies to cardiac myosin were derived from BALB/c mouse lymphocytes as described (8). The antibody used in this study, CCM-52, is in immunoglobulin class IgG-2a and has been shown to react strongly with the heavy chain of mammalian ventricular myosin while not reacting with skeletal myosins (9). Monoclonal IgG was prepared by ammonium sulfate fractionation of ascites fluid of BALB/c mice that had received an intraperitoneal injection of a subcloned line of hybridoma CCM-52. Each rat received an intravenous injection of 9.6 mg of monoclonal IgG (Lowry method, bovine serum albumin as standard) in 0.5 ml of borate-buffered saline (pH 8.4). This quantity of antimyosin was an attempt to provide saturating conditions for labeling the myosin of ischemic cardiac myocytes, given about 15 mg as the entire myosin content of a single rat heart (10). None of the rats displayed any obvious ill effects from this injection over 24 hr of observation.

To determine its clearance rate, sequential serum samples taken from the tail were analyzed for antimyosin titer by using solid-state enzyme-linked immunosorbent assay (ELISA). Diluted samples (25 μl) were applied to myosin-coated microtiter wells and processed with a β-galactosidase hybridoma screening kit (Bethesda Research Laboratories). Quantitative antimyosin titers were scored by reading the absorbance of the developed assay at 405 nm on a Dynatech Microelisa spectrophotometer.

At intervals after LCAO, the ventricles of each heart were cut transversely into five 2-mm-thick slices, which were quick frozen in isopentane chilled in liquid nitrogen. Complete cross sections of the frozen ventricles were cut on a cryostat, dried for 30 min, and sequentially treated as follows: 30 min wash in phosphate-buffered saline, 30 min in fluorescein-conjugated rabbit anti-mouse IgG (Cappel Laboratories, Cochranville, PA), 30 min in phosphate-buffered saline, 5 min in 1.5% paraformaldehyde, and 30 min in phosphate-buffered saline. The sections

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Abbreviations: LCA, left coronary artery; LCAO, LCA occlusion; ELISA, enzyme-linked immunosorbent assay.

were mounted in Elvanol and examined on a Zeiss microscope equipped with a xenon UV lamp and epi-fluorescent optics.

RESULTS

Discrete intracellular localization of antimyosin was detectable by indirect immunofluorescence in zones of ischemia as early as 3 hr after LCAO and antimyosin injection *in vivo*. The appearance of a striated myofibrillar staining pattern (Fig. 1*A*) confirmed both that the sarcolemma of ischemic cells was permeable to immunoglobulin and that antibody bonded specifically to myosin in the myofibrillar A bands. Depending on the duration of ischemia, fluorescent cells formed different patterns of staining. When LCAO was sustained for 3–4 hr, only heterogeneous staining of individual cells appeared in the subepicardium (Fig. 1 *B* and *C*); the subendocardium was un-

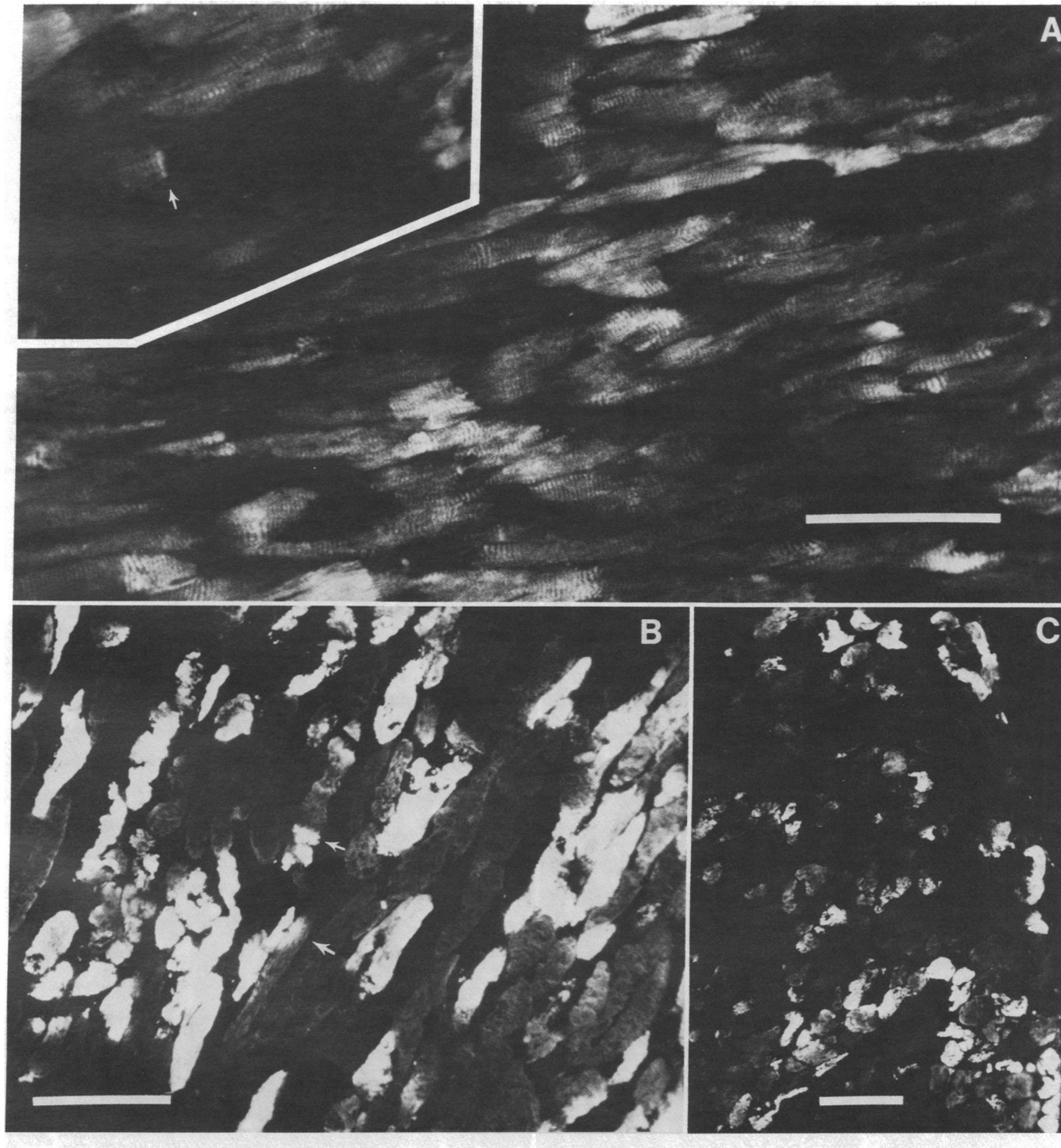

FIG. 1. Photomicrographs of ischemic rat subepicardium labeled *in vivo* with monoclonal antimyosin for 3–6 hr and stained with fluorescein-conjugated anti-mouse IgG. (*A*) From a heart after 6 hr of LCAO. (*Inset*) From the same field as in *A* at higher magnification; the arrow points to an intercalated disc separating two cells of markedly different staining. (*B* and *C*) From a heart 3 hr after LCAO. The arrows in *B* show examples of cells with eccentric staining. Marked heterogeneity of fluorescence is apparent. Note the striated pattern of the fluorescence in cells of *A*. (Bars = 100 μm.)

stained. At 6 hr, while heterogeneous staining persisted in the subepicardium (Fig. 1*A*), immunofluorescent cells clearly outlined the entire transmural extent of the ischemic area. By 24 hr of LCAO, fluorescence within the boundary of the infarct was dense, and heterogeneity was no longer apparent (Fig. 2*A*).

The heterogeneous pattern of cellular staining observed in the ischemic subepicardium of all hearts that had sustained LCAO for 3–6 hr is shown in Fig. 1 *A–C*. Intensely fluorescent cells abutted cells of minimal fluorescence, sometimes across intercalated discs (Fig. 1*A*, arrow). Cells with intermediate levels of fluorescence were also observed. Staining often permeated much or all of the cells. However, certain cells were stained eccentrically (Fig. 1*B*, arrows). The fluorescence in one of these cells (thick arrow) appears to suggest antimyosin spreading out about a presumed defect in the plasma membrane. In contrast to these early patterns, when LCAO was sustained for 24 hr, virtually every cell within the infarct was fluorescent (Fig. 2*A*).

The margins of ischemic zones were sharply outlined by fluorescent cells after both 6 and 24 hr of LCAO (Fig. 2 *A* and *B*). The highly irregular border was clearly defined at each point, with brightly fluorescent cells juxtaposed to cells of only background fluorescence. The convolutions of the border included complex interdigitations of columns of fluorescent and nonfluorescent cells as well as peninsulas of stained cells penetrating unstained tissue (Fig. 2 *A–C*).

Undetectable staining of the endocardial part of the ischemic region early in the course of occlusion would result if insufficient quantities of antimyosin were available because either the blood antibody concentration or the residual circulation into portions of the region was too low. To find out if the antimyosin content of circulating blood was sufficient to label injured cells, the clearance rate of the antibody was determined by measuring its titers in serum samples by ELISA. The change in levels of circulating antibody during 24 hr after LCAO and injection of 9.6 mg of antimyosin is shown in Fig. 3*B* and is contrasted with the levels in a normal rat injected with the same quantity of antibody shown in Fig. 3*A*. High levels of circulating antimyosin remained in both rats, though the titer fell to less than 1/7th in the rat with LCAO. Presumably, this was due to antimyosin binding in the ischemic myocardium. To assess the role of the coronary circulation in the delivery of antimyosin to injured cells, a heart was perfused for 30 min by a solution of antimyosin in oxygenated Krebs–Henseleit buffer (0.2 mg/ml), with the LCA ligature removed after 4 hr of LCAO *in vivo*. The transmural boundary of the ischemic region was clearly delineated, including the subendocardium, though staining in the epicardial half remained heterogeneous.

Binding of antimyosin was observed only in damaged myocytes in the distribution of the occluded LCA. Fluorescence above background was never observed in regions remote from the ischemic area. No fluorescent cells were observed in normal rat hearts examined 24 hr after intravenous injection of antimyosin.

DISCUSSION

This study describes the application of monoclonal antibodies to the analysis of the cellular patterns of evolving myocardial injury by using immunofluorescence. Fluorescent staining is dependent on the entry through abnormally permeable mem-

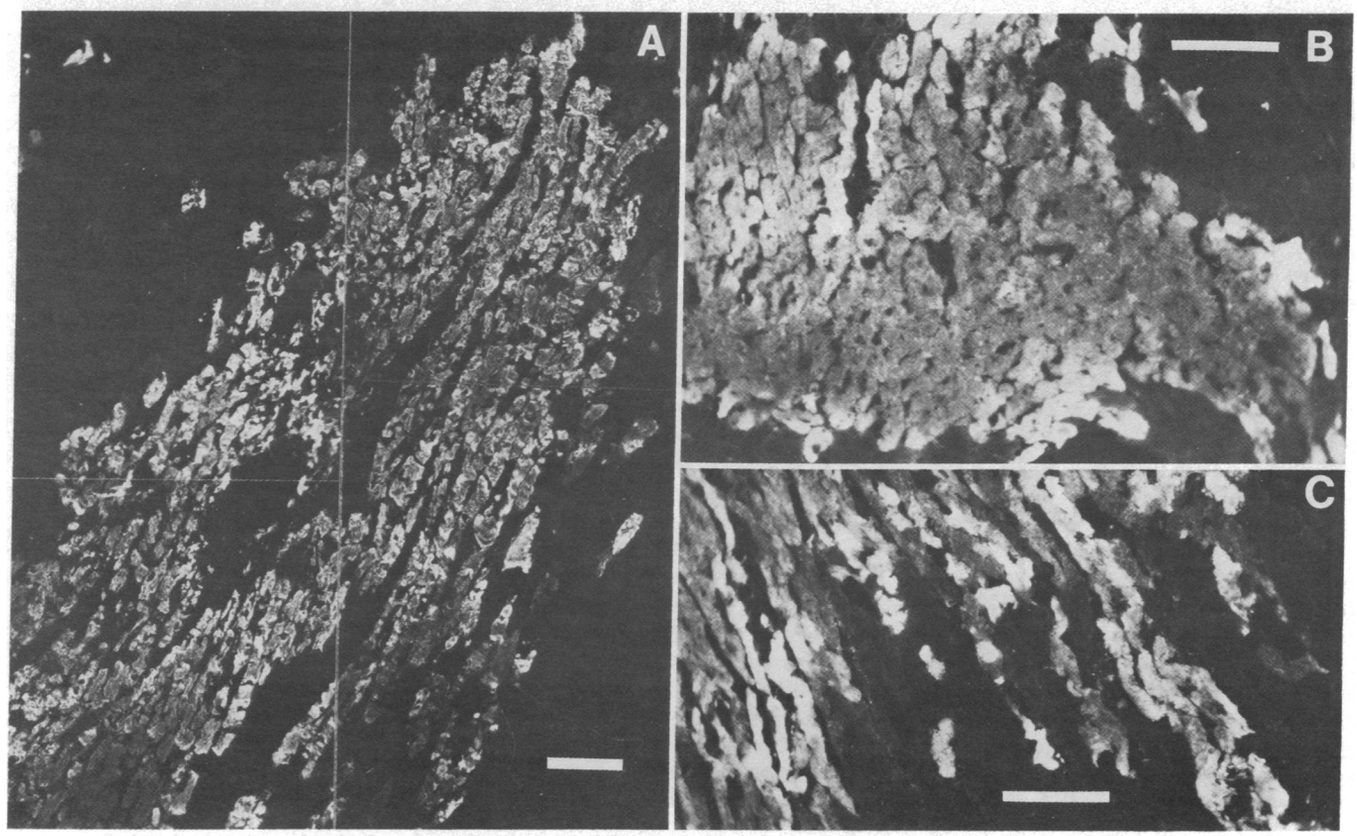

FIG. 2. The border of the ischemic zone. (*A*) A low-magnification composite micrograph along the boundary of ischemic tissue labeled with antimyosin for 24 hr after LCAO. Note the sharply defined region of fluorescent cells, which are completely surrounded by apparently normal cells displaying only background fluorescence. (*B*) A peninsula of fluorescent cells 6 hr after LCAO and injection of antimyosin. (*C*) Detail of the complexity of the border between fluorescent and unstained cells in a heart after 6 hr of ischemia. (Bars = 100 μm.)

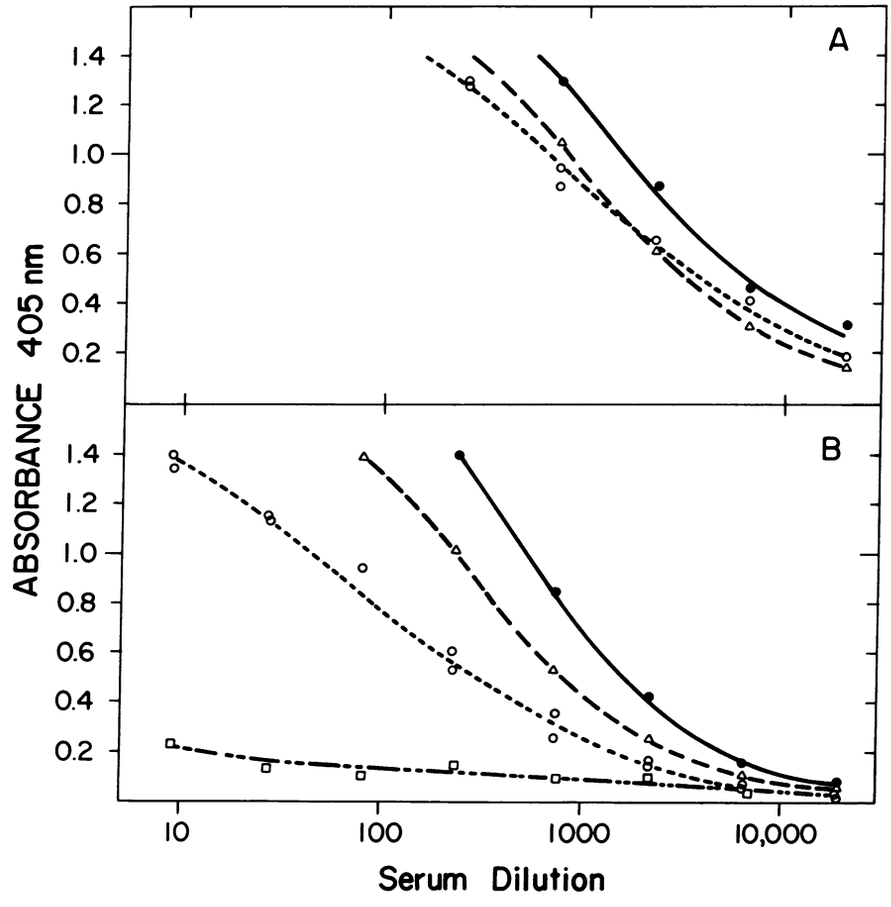

FIG. 3. Circulating antimyosin titers in rats determined by β-galactosidase-conjugated ELISA. Serum samples were taken from rat tails at 2 hr (●——●), 8 hr (△---△), and 24 hr (○----○) after injection of 9.6 mg of monoclonal antimyosin. ELISAs were conducted on serial dilutions of each serum sample. (*A*) Antimyosin clearance in a normal rat without LCAO; (*B*) antimyosin clearance during 24 hr after LCAO and antimyosin injection. The background activity of normal rat serum not injected with antimyosin is given by □—·—□ in *B*.

branes into ischemic cells by antimyosin supplied via the circulation. The appearance of a striated pattern of fluorescence is clear evidence that antimyosin penetrates ischemic cell membranes to bind specifically to myosin in A bands. The limited, eccentric staining seen in some cells (Fig. 1*B*) suggests that the plasma membrane may become leaky only at restricted sites.

Two conceivable ways by which antimyosin is delivered to ischemic cells are (*i*) via collateral circulation and (*ii*) via diffusion from adjacent perfused tissue or blood in the ventricular cavity. Our studies showed that detectable antimyosin did not enter the ischemic subendocardium until 6 hr after sustained LCAO. Four hours was insufficient, unless the ligature was removed, when the ischemic part of the subendocardium was well defined by fluorescence. In contrast, inhomogeneous staining of individual cells in the subepicardium was routinely observed after 3–6 hr of LCAO. These findings correspond to the proportionately more severe reductions in regional blood flow that occur with coronary occlusion in the subendocardium versus the subepicardium. Reimer and Jennings (11) presented evidence in dogs that infarction begins in the subendocardium and extends into the subepicardium over 3–6 hr, the ultimate extent being determined mainly by collateral circulation. Regional blood flows measured 20 min after coronary occlusion in these dogs averaged 3% of nonischemic flow in the subendocardium and 17% in the subepicardium, where they were more variable (range, 1–41%). Thus, in our experiments, it appears that with LCAO, antimyosin is delivered to injured cells in the subepicardium via existing collateral circulation, whereas in the subendocardium, where severe reductions in blood flow exist, diffusion over longer distances is the main route and requires about 6 hr.

The pattern of heterogeneous staining, visible only in the epicardial half in the early stages of ischemia, suggests that collateral circulation in the subepicardium may be sufficient to temporarily support some cells but not others. However, several mechanisms might account for this appearance: (*i*) the distribution of perfused and unperfused capillaries, (*ii*) gradients in antimyosin concentration diffusing into the area, and (*iii*) heterogeneity in cellular resistance to ischemia resulting from different properties of individual myocytes. Regarding the first mechanism, by using reduced NADH fluorescence, Chance and colleagues demonstrated a macroscopic heterogeneity of anoxic and normoxic zones in hypoxic and ischemic rat hearts (12, 13). The smallest anoxic zone was some "several hundred microns" in diameter. Because that diameter exceeds intercapillary distances by a factor of about 10, they postulated that the regulation of coronary perfusion in ischemic and hypoxic states resides in arterioles rather than capillaries (12). We surveyed the distribution of fluorescent cells over the entirety of each cross section of the left ventricles in our study. We did not observe a pattern that would clearly correspond to the macroscopic array of anoxic and normoxic zones described by Chance and colleagues, possibly because zones of NADH fluorescence expanded and became confluent with increasing severity of hypoxia (12, 13). But, as the average width of a rat cardiac myocyte is about 11 μm (14), the heterogeneous fields seen in

Fig. 1 would span a number of capillaries because the mean intercapillary distance in the rat varies between 11 and 17 μm with oxygenation (15). Thus, the patterns of fluorescence that we do observe are in agreement with a conclusion that ischemic flow is partitioned at the arteriolar level.

Another possibility is that heterogeneous fluorescence is due to diffusion gradients of antimyosin. Although such gradients undoubtedly exist within the ischemic region, they would have to be exceedingly steep to account for the entire distribution of cells in heterogeneous areas, where intensely stained cells abut cells of negligible staining and precipitous drops of staining intensity occur at intercalated discs (Fig. 1). Furthermore, heterogeneity persists within the confines of the ischemic area defined by immunofluorescence when perfusion is effected by removing the occluding ligature.

A third mechanism that may explain heterogeneity is that certain myocytes in a field of ischemia are more resistant to such deprivation than others. Cardiac myosin Ca^{2+}-activated ATPase and speed of shortening vary with isomyosin composition (16–18). The distribution of different isoenzymes of myosin among individual cardiac myocytes in several species, including the normal adult rat, is heterogeneous (19, 20). Moreover, Baumgarten *et al.* (21) reported two populations of cells differentiated by their potassium equilibration potentials and their intracellular potassium activities in hypoxic guinea pig papillary muscle. Jennings and co-workers noted that reversible and irreversible ultrastructural changes were not distributed uniformly among cells in ischemic regions (1, 2). Thus, the adaptability of cardiac myocytes to ischemic stress may be related to particular properties determined by such factors as their isomyosin composition.

Recent investigations have indicated that the junction of ischemic with normal myocardium is discrete (13, 22, 23). In our study, the boundary between presumably irreversibly injured ischemic cells (fluorescent) and surrounding cells with presumably intact cell membranes (background fluorescence) is extremely sharp, both at 6 and 24 hr after LCAO. The complexity of this boundary supports the concept advanced by Kirk and colleagues that the interdigitation of normal and infarcted tissue at the border is a consequence of corresponding intermeshing of capillary beds supplied by patent and occluded arteries (22).

We conclude that staining ischemic cardiac myocytes by means of monoclonal antibody to myosin is a highly specific and sensitive technique for defining the patterns of developing cellular injury. We have demonstrated explicit visualization of presumably irreversibly injured cells via penetration of their cell membranes by antimyosin. Applied early in the course of ischemia, the technique has provided visual evidence of a heterogeneous population of cells, divergent in their response to ischemia. Although the exact mechanisms accounting for this heterogeneity are not yet established, it may be that certain cardiac myocytes are more resistant to ischemia than others, by virtue of specific characteristics such as their enzymatic and contractile properties. The convoluted border between cells penetrated by antimyosin and cells with apparently intact cell membranes is explicitly defined, cell by cell, whether at 6 or 24 hr after occlusion. The determination of the blood clearance rate for antimyosin (Fig. 3) may also be useful in providing a means for estimation of the extent of myocardial necrosis resulting from coronary occlusion.

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