American Journal of Pathology, Vol. 148, No. 6, June 1996

Acute Inflammatory Reaction after Myocardial Ischemic Injury and Reperfusion

Development and Use of a Neutrophil-Specific Antibody

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Reperfusion of the infarcted canine myocardium after 1 hour of ischemia is associated with an acute inflammatory infiltrate at the border of the infarct. In this paper, we demonstrate that early margination and emigration of neutrophils originate in thin-walled (~5-μm) venous cisterns that average 200 μm in length and vary from 10 to 70 μm in width and show strong constitutive expression of both ICAM-1 and P-selectin; this class of vessels (venous cisterns) appears to be a unique feature in heart. A monoclonal antibody (SG8H6) with specificity for canine neutrophils was developed that allowed much more sensitive immunohistochemical detection of neutrophils in tissue and allowed us to follow tissue infiltration with time. Samples from 1 hour of reperfusion revealed dense margination and substantial emigration of neutrophils associated with the venous cisterns and collecting venules. By 2 hours, there was intense local emigration to the extravascular space between cardiac myocytes. By 3 hours, the infiltrate extended deeper into the infarct, and there was a continuous border zone of neutrophil infiltration that overlapped a region where intact cardiac myocytes strongly expressed ICAM-1 mRNA and extended into the necrotic tissue. At later times, neutrophil migration into infarcted tissue continued to progress. Neutrophil transmigration into reperfused myocardium is more extensive than previously described, and its extravascular distribution during early reperfusion is primarily in the viable border zone of the myocardium where myocyte ICAM-1 mRNA is found. These data are compatible with the hypothesis that extravascular neutrophils may participate in reperfusion injury. (Am J Pathol 1996, 148:1957–1969)

Ischemia and reperfusion of myocardium result in localization of neutrophils in the ischemic regions,[1,2] which occurs within the initial few hours of reperfusion.[3,4] The timing of this localization corresponds with the appearance of C5a-dependent chemotactic activity in the lymph draining the reperfused tissue.[5,6] Emigration of neutrophils into the interstitium is evident both histologically[7,8] and by the fact that neutrophils are found in reperfusion cardiac lymph.[5] This localization apparently causes tissue injury beyond the ischemic insult, as interventions in several animal species that reduce either the number of neutrophils, their adhesion, their function, or their leukotactic activity significantly reduce the amount of tissue damaged.[7,9–17] The anatomic distribution of neutrophil localization throughout the reperfused tissue has not been precisely defined. Early reports on experimental myocardial infarction[18–20] described infiltration of polymorphonuclear neutrophils, particularly after temporary coronary occlusion and reperfusion, but considered the inflammatory reaction to be relatively inconsequential and did not describe an association of inflammation with a particular vessel type. There is evidence for trapping of neutrophils within small vessels,[21] particularly in the severely ischemic regions, and there is evidence for localiza-

Supported by National Institutes of Health grants HL-42550 and HL-47163.

Accepted for publication February 5, 1996.

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tion in regions of partial ischemia.[4] In the present study we describe the development and application of a neutrophil-specific monoclonal antibody that aids in identification and quantitation of the infiltration of neutrophils into tissue and describe an association of the acute inflammatory infiltrate with a class of small veins that strongly express both ICAM-1 and P-selectin.

We have recently observed a border zone consisting of apparently viable myocytes surrounding regions of irreversibly injured myocytes in canine hearts subjected to 1 hour of ischemia and various times of reperfusion that is clearly demarcated by the expression of ICAM-1 (CD54) mRNA.[22] Normal myocytes show no constitutive expression of ICAM-1, and irreversible myocyte injury due to severe ischemia of 1 hour duration is apparently sufficient to prevent myocytes from expressing ICAM-1. This zone appears to represent an area where intermediate reduction in blood flow initiates events that stimulate synthesis of ICAM-1 by the myocytes. The pathogenic significance of this region of ICAM-1 mRNA expression is unknown, but our recent studies of neutrophil interactions with isolated cardiac myocytes[23] indicate that neutrophils can kill cardiac myocytes through an adherence-dependent mechanism involving ICAM-1[24,25] on the myocyte and CD11b/CD18 (Mac-1) on the neutrophil.[26] For such a mechanism to operate *in vivo*, neutrophils must be present at the time and place myocytes are expressing ICAM-1. Such a co-localization has not been clearly shown.

In the present study, we provide evidence in support of the hypothesis that neutrophils can emigrate into reperfused, viable myocardial tissue in the area where myocytes express ICAM-1.

Materials and Methods

Animal Model

The experimental procedures used have been described previously.[27] Healthy mongrel dogs of both sexes weighing 15 to 25 kg were anesthetized by intravenous injection of methohexital (10 mg/kg), intubated, and ventilated and given the inhalational anesthetic Isoflurane. A midline thoracotomy was performed, and a hydraulically activated occluding device and a Doppler flow probe were placed around the proximal left circumflex coronary artery just proximal or just distal to its first branch. In most animals, the chest was closed and they were allowed to recover for at least 72 hours before coronary artery occlusion, which was performed while the animals were sedated with intravenous pentazocine (0.1 to 0.2 mg/kg) but awake. In a few animals, the major lymphatic vessel of the heart was also ligated and the other regional lymphatics were ligated at the time of surgery to collect cardiac lymph for study. Another group of animals was studied without prior surgical preparation, occluding the coronary artery under anesthesia, to test for possible priming or enhancement by prior surgery of the inflammatory response in the heart. Ischemia was induced by inflating the occluder around the circumflex coronary artery until the flow probe indicated zero mean blood flow. After 1 hour, the cuff was deflated to allow reperfusion of the previously ischemic myocardium. Arterial blood pressure, heart rate, circumflex blood flow, and electrocardiogram (limb lead II) were recorded continuously. Radioactive microspheres were injected into the left atrium before coronary artery occlusion, 15 minutes after coronary artery occlusion, and 15 minutes after beginning reperfusion, and 2 minutes after injection, a reference sample of arterial blood was obtained. After 1 hour of coronary artery occlusion, tissue was obtained with no reperfusion or after reperfusion of whole blood for 1, 2, 3, 6, or 24 hours. We found that 1 hour of circumflex coronary artery occlusion was sufficient to produce a zone of irreversible injury that nearly filled the posterior papillary muscle in most animals. Tissue was also obtained after 1, 3, 4, or 6 hours of coronary artery occlusion without reperfusion. Control myocardium was obtained from previously untreated animals and from occasional animals in which the coronary artery had been occluded but no infarct developed because of collateral circulation. Hearts were stopped by infusion of saturated KCl and excised.

Histological and Immunocytochemical Methods

Hearts were sectioned parallel to the base into four or five transverse rings. Adjacent transmural sections were obtained through the anterior and posterior papillary muscles and from the septum and were fixed in phosphate-buffered 10% formalin or rapidly frozen in OCT using isopentane cooled with liquid nitrogen. Additional samples were taken from the mid-myocardium in a plane parallel to the surface of the heart. Six samples of myocardium were obtained from the anterior and posterior papillary muscle regions adjacent to the histological tissue samples, weighed, and fixed in phosphate-buffered formalin for estimation of regional blood flow by measurements of their radioactivity as described previously.[28–30]

Histological sections 6 μm thick were cut from paraffin-embedded tissue and stained with hematoxylin and eosin (H&E). Cryostat sections 6 μm thick were fixed for 10 minutes in acetone and air dried. Immunohistochemical staining was carried out using monoclonal antibodies CL18/1D8, which binds canine ICAM-1 (CD54), and MD3, which binds canine P-selectin (CD62P), both of which were developed in the Leukocyte Biology Laboratory, Baylor College of Medicine,[24,31] monoclonal antibody R15.7, which binds canine CD18[23] (provided by Robert Rothlein, Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT), and monoclonal antibody SG8H6,[32] a neutrophil-specific antibody described below. A nonbinding, isotype-matched monoclonal antibody was used in each staining procedure as a control, in addition to controls omitting the primary antibody and biotinylated link reagent. An additional monoclonal antibody, 2F9, which reacts with canine platelets but not endothelial cells, was used in conjunction with P-selectin staining to discriminate platelet aggregates from vascular expression of P-selectin. Cryostat sections were pretreated for 30 minutes with 1% sodium metaperiodate, for 30 minutes with 0.1 mol/L ammonium chloride, and for 15 minutes with 1% horse serum. Sections were incubated overnight with primary antibody at 4°C in humidified chambers. In addition, paraffin sections were immunostained using commercial rabbit antisera to lysozyme and monoclonal antibody SG8H6, both of which provided intense staining of neutrophils. Antibody-binding sites were labeled using an avidin-biotin-peroxidase complex (ABC) method (Vector Laboratories, Burlingame, CA), using diaminobenzidine as substrate with enhancement of the intensity of the reaction product by brief immersion in 1% osmium tetroxide. Primary antibody was normally used at a concentration of 10 μg/ml. To estimate the relative degree of expression of antigen in different tissue sites, multiple dilutions of antibody were used, ranging from 0.01 to 20 μg/ml.

Development and Characterization of SG8H6

SG8H6, a monoclonal antibody recognizing canine neutrophils, was prepared by immunizing BALB/c mice with isolated canine neutrophils, and hybridomas were produced as previously described.[23] Clones were initially screened for binding to canine neutrophils using flow cytometry of isolated neutrophils and secondarily determined to be neutrophil specific using whole blood preparations for flow cytometry and cell sorting. Canine whole blood was labeled with fluorescein isothiocyanate (FITC)-labeled SG8H6. The number of binding sites for this labeled antibody preparation was estimated using the Quantum Simply Cellular technique (Flow Cytometry Standards Corp., San Juan, PR). The specificity for neutrophils was also determined by cell sorting whole blood preparations after labeling with FITC-tagged SG8H6. Erythrocytes were hypotonically lysed and cells were evaluated on a Coulter Epics cell sorter using a gate that included all leukocytes. Two peaks of fluorescence were found, one with very low binding having a mean fluorescent intensity (MFI) of <10 and another with an MFI of >900. Cells from the high binding peak were collected, and cytospin smears were prepared and stained (Neat Stain, Midlantic Biomedical, Paulsboro, NJ) for differential counts. From the initial immunization and cloning, three hybridomas specific for canine neutrophils were obtained. SG8H6, in contrast to the other clones, demonstrated the ability to bind to leukocytes in sections from formaldehyde-fixed, paraffin-embedded tissues.

Morphometry of the Inflammatory Infiltrate

The density of the infiltrate of neutrophils within irreversibly injured myocardium was measured near the edge of the infarct and in an adjacent field deeper into the infarct, as a means of observing the progressive accumulation of neutrophils in a zone of increasing width adjacent to the edge of the infarct. Irreversibly injured myocytes were identified by the presence of distinct contraction bands. Sections stained with monoclonal antibody SG8H6 were analyzed using a video microscope and Optimas image analysis software (BioScan, Edmonds, WA). Using a 25×, 0.6 numerical aperture (n.a.) objective, a field was selected that measured 250 × 200 μm. Initially, the field was centered over the edge of the infarct and then moved to the adjacent field within the infarct for measurement A and to the adjacent field deeper into the infarct for measurement B. Field A thus extended from 125 to 375 μm inward from the border of the infarct and field B from 375 to 625 μm. The images were stored and the density threshold adjusted to discriminate neutrophils from nuclei and other dense objects; the discrimination was confirmed visually. Counts were made automatically, and 20 fields were analyzed per animal.

The number of neutrophils surrounding blood vessels was estimated using Optimas image analysis software. Using histological sections stained with SG8H6 and very lightly counterstained with eosin, all

arteries and veins were included that were associated with neutrophils and in which the endothelial lining was clearly visible. The length and width of the vessel profile were measured, and the number of neutrophils inside and outside the endothelial layer was recorded in a standard field measuring 160 × 200 μm. This was done using a 40×, 0.65 n.a. objective and reverse video mode, adjusting the illumination to provide a mean luminance of 50 ± 1, capturing the image, and automatically outlining structures with luminance values >100, including unstained inclusions, which represent nuclear outlines. This procedure consistently outlined neutrophils. The mean area of a neutrophil inside a vessel was determined by measuring 50 solitary neutrophils inside vessels and found to be 25.95 μm^2. Similarly, the mean area of a solitary neutrophil outside a vessel was found to be 16.02 μm^2. The total area outlined inside and outside each vessel was measured and divided by the appropriate mean area of a single neutrophil to estimate the number of neutrophils inside and outside each vessel. The data were collected and analyzed in Microsoft Excel worksheets using linear regression analysis and Student's *t*-test for unequal variances. To estimate the density of the inflammatory infiltrate associated with each vessel type, images were analyzed using Optimas software to count the number of neutrophils in fields measuring 50 × 50 μm sampled within dense infiltrates surrounding venous cisterns, around all arteries in the section, and in representative areas within the zone of irreversible injury, which contained only capillaries.

Detection of ICAM-1 mRNA in the Ischemic Border Zone

Digoxigenin-labeled probes were prepared by *in vitro* transcription from a linearized template following the method recommended by Boehringer Mannheim in the Genius RNA probe labeling kit, as described previously.[22] A 150-bp fragment of canine ICAM-1 cDNA taken from the published sequence was subcloned in both orientations into P-bluescript II SK+ and PGEM-3 so that use of T7 polymerase would result in the generation of single-stranded antisense and sense RNA probes. Both RNA probes were precipitated with glycogen and sodium acetate, washed with 70% ethanol, and resuspended in diethyl pyrocarbonate (DEPC)-treated water. Both probes were verified by hybridization and detection on Southern blots and Northern blots on nylon membranes.

In situ hybridization was carried out using deparaffinized sections following the procedure from Boehringer Mannheim with the Genius system as previously described in detail.[22] Briefly, hybridization was performed overnight at 42°C using 500 ng/ml ICAM-1 sense or antisense probe in fresh prehybridization buffer. Slides were washed stepwise in decreasing concentrations of standard saline citrate from 2× to 0.5×, and immunological detection of hybridized probe was carried out using a 1:500 dilution of anti-digoxigenin antibody. The slides were then incubated overnight at room temperature in the dark with the color solution from the Genius detection kit containing nitroblue tetrazolium.

Results

Histopathology

Initially, the response to ischemic injury was studied using conventional histological methods. At all intervals of reperfusion studied, regions of irreversible injury of cardiac myocytes were readily identifiable in longitudinal sections by the presence of prominent contraction bands, increased eosinophilia, and myocyte swelling. These regions of necrosis were situated near the endocardial surface but separated from it by a zone of intact myocytes as previously described.[22] Hemorrhage was only rarely present, and blood vessels and their endothelial linings appeared intact. In control tissue and in tissue subjected to ischemic injury for 1 hour without reperfusion, the tissue retained a normal light microscopic appearance, no inflammatory reaction was seen, and there was no accumulation of neutrophils within the blood vessels (data not shown). In tissue injured by 1 hour of ischemia followed by 2 or 3 hours of reperfusion, numerous neutrophils were present at the edge of the region of necrosis, surrounding venous structures that had thin walls and elongated profiles, as described below. After only 1 hour of reperfusion, neutrophils were present around similar blood vessels but were less prominent. Margination of neutrophils was prominent after reperfusion for 1, 2, 3, 6, or 24 hours. To quantitate the infiltrate of neutrophils more accurately, we developed a neutrophil-specific antibody (see below), which revealed that the degree of infiltration is underestimated by conventional histological study. An occasional animal was excluded from the study because small foci of healing injury were seen that were thought to be due to surgical implantation of flow probes and occluders. Except for reactive epicardial changes, no differences in the extent of the inflammatory response were apparent in animals that were surgically prepared several days before coronary artery

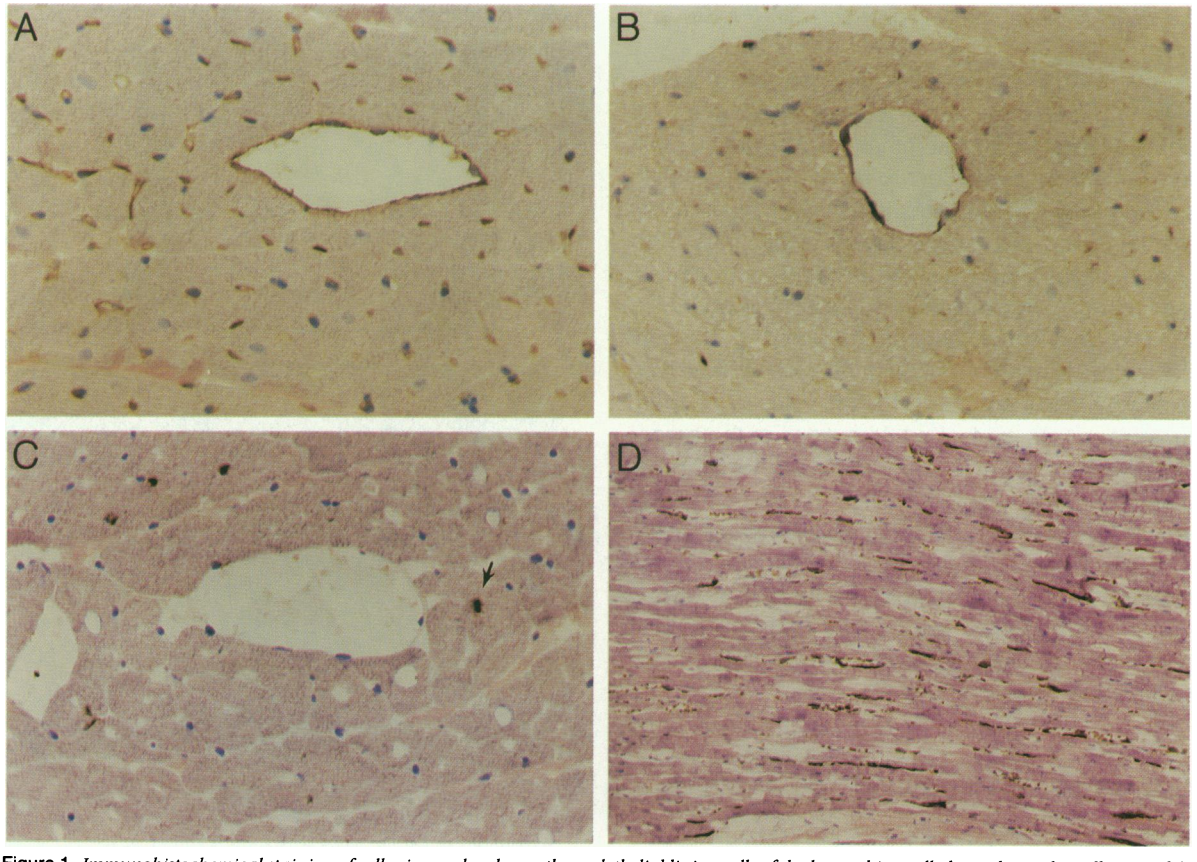

Figure 1. *Immunohistochemical staining of adhesion molecules on the endothelial lining cells of the large, thin-walled venules and small veins of the heart. These vessels generally appear ovoid or elongated in section and show distinct staining for ICAM-1 and for P-selectin even at low concentrations of primary antibody.* **a:** *Immunostain for canine ICAM-1 in control tissue using monoclonal antibody CL18/1, producing a brown reaction product. The tissue is lightly counterstained with H&E. Both the large ovoid venule and capillaries stain intensely. The vein or venule measures approximately 35 × 100 μm in cross section. Original magnification, × 700.* **b:** *Immunostain for P-selectin using monoclonal antibody MD3. This pattern of staining persists in the zone of irreversible myocyte injury. Original magnification, × 700.* **c:** *Immunostain using platelet-specific monoclonal antibody 2F9; same specimen as* **a.** *No staining is seen in the large venule, although rare platelets within capillaries are stained* (**arrows**). *Original magnification, × 700.* **d:** *Immunostain using platelet-specific monoclonal antibody 2F9; zone of infarction after 1 hour of circumflex coronary artery occlusion and 3 hours of reperfusion. Numerous small clumps of platelets are stained within capillaries. Original magnification, × 280.*

occlusion as compared with animals subjected to coronary artery occlusion under anesthesia.

Immunostaining of ICAM-1 and P-Selectin

Immunostaining for ICAM-1 utilizing the antibody CL18/1D8 at concentrations between 5 and 20 μg/ml revealed strong continuous staining of the endothelial layers of all blood vessels including capillaries, arterioles, venules, arteries, and veins. With low concentrations of primary antibody (less than 1 μg/ml), arterial and venous endothelium stained very well, but smaller vessels did not stain. A distinct population of interstitial veins stained for ICAM-1 at such low concentrations of antibody, as shown in Figure 1a, which we refer to as venous cisterns. These vessels varied from 10 to 70 μm in width, had thin walls approximately 5 μm thick, and appeared highly elongated, ranging from 50 to more than 500 μm in length, averaging 200 μm in length. They were often present in delicate loose connective tissue septa but were also seen within the myocardial tissue with no surrounding connective tissue. In longitudinal sections of myocytes, these veins extended in the same direction as the myocytes; where myocytes were seen in cross section, they extended in various directions around clusters of myocytes. Somewhat smaller venous channels that were surrounded by myocardial cells showed similar staining and are interpreted as collecting venules.

Staining of P-selectin was prominent in the venous cisterns and in collecting venules in both experimental and control tissue (Figure 1b), and these vessels did not stain with anti-platelet monoclonal antibody 2F9. In contrast, positive staining for P-selectin was not seen in capillaries except within the infarct, where staining with the anti-platelet antibody 2F9 was also demonstrated. This suggested that the P-selectin staining observed in small vessels in the infarcts resulted from platelet aggregates rather than

from vascular expression of P-selectin (Figure 1, c and d).

After 1 hour of circumflex coronary artery occlusion and 1 hour of reperfusion, leukocytes were seen accumulating within the lumens of the venous cisterns in the area adjacent to the border separating apparently viable myocytes from those showing contraction band necrosis. The leukocytes often appeared to be lined up side-by-side in contact with the venous endothelium. Penetration through the walls of the venous cisterns and into the adjacent connective tissue surrounding the vessels was seen in slightly less than one-half of the veins in the area. Emigration was evident on both sides of the border between necrotic myocardial cells and visually normal myocytes. Venous cisterns and collecting venules appeared to be the principal sites for leukocyte emigration. They constitutively expressed both P-selectin and ICAM-1, which have been shown *in vitro* to play important roles in leukocyte margination and transmigration, respectively, with human[33–35] and canine[26,32,36] neutrophils.

Characterization of SG8H6

To help to identify neutrophils in histological sections, we developed a monoclonal antibody suitable for immunolocalization of canine neutrophils in sections of fixed, paraffin-embedded tissues. As shown in the flow cytometric analysis of FITC-labeled monoclonal antibody SG8H6 in canine whole blood, a single peak of intense binding is detected (Figure 2). When this peak was collected by cell sorting, 98% of the cells were neutrophils with the remaining 2% being either eosinophils or lymphocytes. No monocytes were found. The mean number of binding sites for SG8H6 on neutrophils was 990,000/cell, whereas the number of binding sites on other leukocytes never exceeded 3000/cell, a value near the limit of resolution of the methods employed. Thus, although the antigen recognized by this antibody may be present at very low levels on other leukocytes, the apparent copy number on neutrophils is ~1000-fold greater.

Immunostaining of Neutrophil Infiltration with SG8H6

Immunostaining of reperfused myocardium with monoclonal antibody SG8H6 demonstrated that many neutrophils were present in the interstitium around venous cisterns and collecting venules near the edge separating necrotic from intact myocytes.

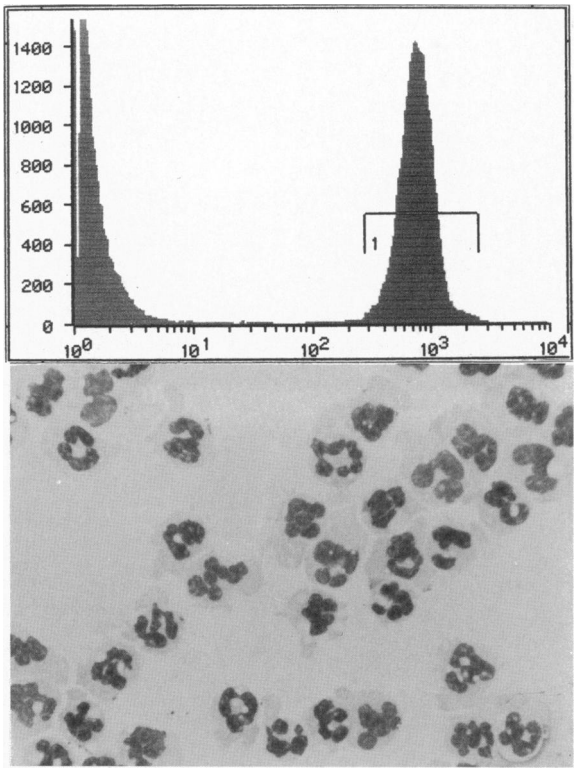

Figure 2. *Binding specificity of monoclonal antibody SG8H6. The flow cytometric pattern of binding of FITC-labeled SG8H6 to buffy coat preparations of canine blood leukocytes is shown in the histogram. Two peaks are evident: one with MFI of < 10 and one with MFI of 980. The second peak was collected by fluorescence-activated cell sorting, and cytospin preparations were made and stained as shown in the photomicrograph.*

Conventional histological methods revealed only limited infiltration of neutrophils into the myocardium after 1 hour of reperfusion (Figure 3a). Staining with SG8H6 allowed detection of migrating neutrophils that were present in the tissue in elongated form and were not recognizable as neutrophils with conventional H&E staining alone. To evaluate the effectiveness of SG8H6 staining, two serial sections were prepared from the posterior papillary muscle after 1 hour of reperfusion, one stained with H&E, the other with SG8H6 (Figure 3, a and b). The number of recognizable neutrophils within a 40× field around 10 small veins was counted on each section. The average number of neutrophils counted was 39.4 on the H&E-stained slide and 74.5 on the SG8H6-stained slide. Thus, immunostaining with SG8H6 allows detection of infiltrates of neutrophils that are not easily recognized in conventional sections and reveals more extensive tissue infiltration. In addition, neutrophils can be recognized and counted easily at low magnification after staining with SG8H6, whereas high magnification is required to recognize

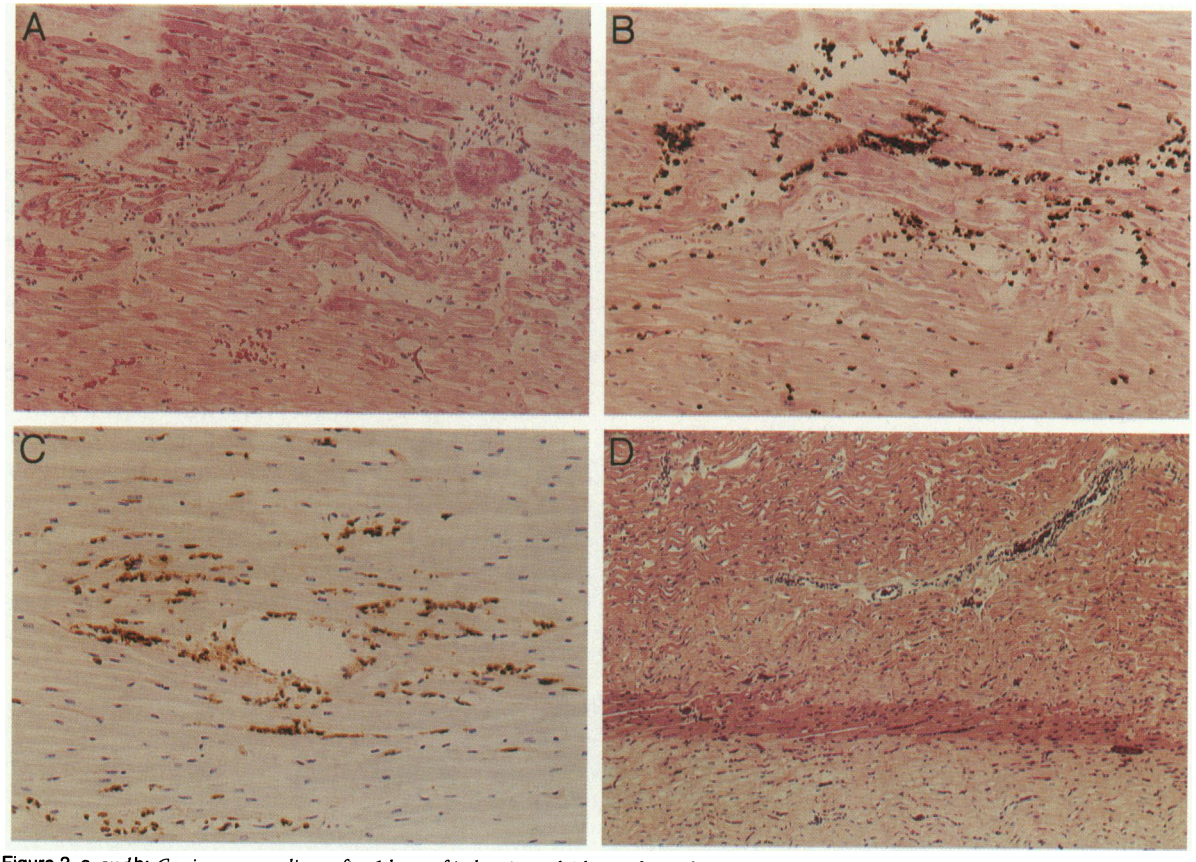

Figure 3. a *and* b: *Canine myocardium after 1 hour of ischemia and 1 hour of reperfusion.* a: *Conventional staining with H&E.* b: *Immunostaining of the adjacent serial section with monoclonal antibody SG8H6. Significantly more neutrophils can be recognized by virtue of specific immunostaining.* c: *Neutrophil emigration after 2 hours of reperfusion; immunostaining with SG8H6.* d: *Tissue subjected to 3 hours of coronary artery occlusion without reperfusion. The border of the zone of irreversible injury is not accompanied by any significant inflammatory infiltrate. Original magnification, ×280.*

neutrophils in H&E-stained sections. Transmigration of neutrophils during the first hour of reperfusion is much more extensive than had been appreciated.[17] By 1 hour of reperfusion (Figure 3b), many neutrophils were still clustered immediately outside the vascular wall, but there was also significant infiltration in the adjacent interstitium.

After 2 hours of reperfusion, foci of intense interstitial acute inflammatory infiltration surrounded the venous cisterns that were located in a narrow band on the border between intact cells and cells exhibiting contraction band necrosis (Figure 3c). Most venous cisterns were surrounded by 5 to 20 neutrophils, although some exhibited cuffs of several hundred neutrophils extending into the adjacent interstitium between myocytes for variable distances of 50 to 300 μm. Morphometric estimation indicated a mean number of neutrophils outside venous cisterns of 97.98 ± 12.84 (SEM). There was an apparent tendency for smaller venous cisterns to be associated with more infiltrating neutrophils, but linear regression analysis did not show a statistically significant association with vein width. The counts of neutrophils outside and inside veins are shown in Table 1. By this measure, the infiltration of neutrophils was maximally intense after 2 hours of reperfu-

Table 1. *Morphometric Estimates of the Number of Neutrophils Outside and Inside Venous Cisterns*

	1 hour	2 hours	3 hours
Outside venous cisterns	28.32 ± 3.69	97.98 ± 12.84*	71.02 ± 6.50†
Inside venous cisterns	7.85 ± 1.07	17.41 ± 2.69*	20.18 ± 5.01*

Results are based on immunostaining with neutrophil-specific monoclonal antibody SG8H6 and assessment of an area measuring 160 × 200 μm centered on each vessel. The times shown are hours of reperfusion after 1 hour of circumflex coronary artery occlusion. The results are shown as mean ± SEM.

*$P < 0.05$ compared with the values at 1 hour.

†$P < 0.05$ compared with the values at 1 and 2 hours.

Table 2. *Number of Neutrophils in Myocardial Tissue after 1 Hour of Circumflex Coronary Artery Occlusion and the Specified Interval of Reperfusion*

Reperfusion (hours)	n*	Distance† 250 μm#	Distance† 500 μm
1	8	25.13 ± 4.6	12.00 ± 2.7
2	4	23.75 ± 6.9	23.50 ± 6.4
3	9	46.11 ± 7.1‡	30.89 ± 5.1‡
24	2	68.00 ± 8.9‡	70.00 ± 5.0‡

Data are presented as mean ± SEM.
*Number of animals evaluated.
†Average distance from the edge of the border zone into the severely ischemic myocardium, indicating the midpoint of a 200 × 250 μm area measured.
‡$P < 0.05$ compared with the values at 1 hour.

sion. The density of the neutrophilic infiltrate around venous cisterns was estimated at 11.96 ± 0.78 polymorphonuclear neutrophils/1000 μm^2, significantly greater than that around arteries (2.49 ± 1.15) or capillaries (1.34 ± 0.24). At later times, more neutrophils had migrated further into the infarct (Table 2) and neutrophil density was increased.

After 3 hours of reperfusion, the greatest intensity of neutrophils was around the venous cisterns just inside the necrotic area and among the intact myocytes adjacent to this area (Figure 4a). There was a perivascular distribution and also a diffuse interstitial infiltrate between the myocytes. Both of these processes showed peak intensity at the border between abnormal and intact myocytes. Because of diffuse filling in of spaces between venous cisterns, the total number of neutrophils in the tissue is greater at 3 hours of reperfusion than at 2 hours. Figure 4b demonstrates a zone at the border of an early infarct in which the interstitium between each myocyte contained rows of neutrophils. The zone of neutrophil infiltration extended from the edge of the region of myocyte necrosis inward for a distance of 0.5 to 1.0 mm. Veins up to 0.5 mm outside the zone of myocyte necrosis were also surrounded by infiltrating neutro-

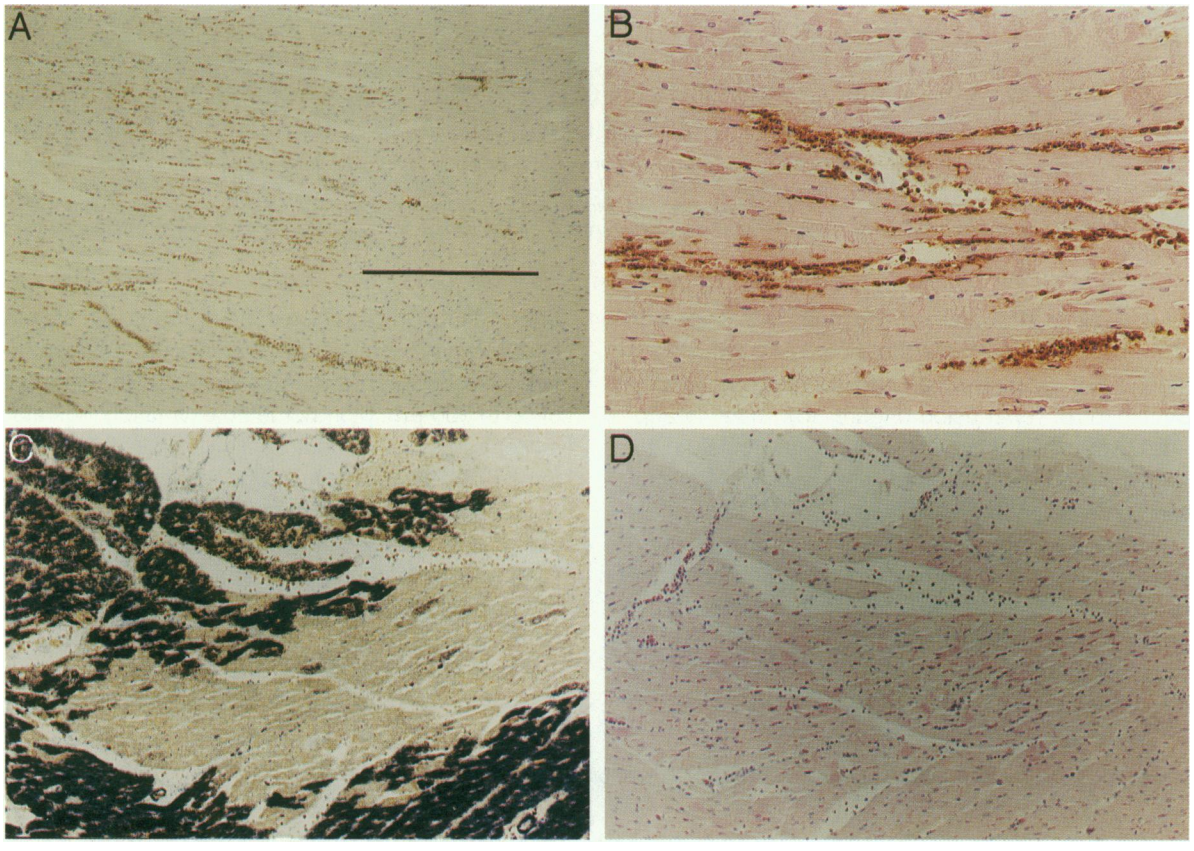

Figure 4. *Canine myocardium after 1 hour of ischemia and 3 hours of reperfusion.* **a** *and* **b**: *Immunostaining with SG8H6. Neutrophils are seen emigrating at vessels of the type shown in Figure 1, a and b. At low magnification* (**a**), *the pattern of neutrophil infiltration can be seen. The right third of the figure reveals intact myocardial tissue, whereas the left two-thirds represents an infarct, identified by the presence of contraction bands. In the center, neutrophils are lined up between myocytes. In the adjacent intact tissue on the left, occasional clusters of neutrophils are seen around small vessels. The scale bar in the figure represents a distance of 500 μm. Original magnification, ×104. At higher magnification* (**b**), *extensive infiltration of neutrophils can be seen between myocytes. The photomicrograph in* **c** *shows* in situ *hybridization with the anti-sense ICAM-1 riboprobe showing the zone between necrotic and viable tissue. The dark blue color reaction localizes ICAM-1 mRNA to the cardiac myocytes. H&E staining of the adjacent serial section* (**d**) *shows extensive leukocyte infiltration in the same region. Original magnification, ×280* (**b** *to* **d**).

phils that extended between viable myocytes in the vicinity of the venous cisterns penetrating the normal zone. There was also an increase in the number of single neutrophils within the capillaries, which was greatest within the zone of necrosis. In nonischemic control tissue from the same animals, there was no inflammatory reaction except for a mild increase in the number of neutrophils scattered within capillaries. When tissue was injured by 3 hours of permanent coronary artery occlusion without reperfusion, the border of the infarct was visible, but no significant inflammatory infiltrate was seen (Figure 3d). After 6 hours of reperfusion, there was a broad zone at the border of the region of contraction-band necrosis, which was diffusely infiltrated by neutrophils. At 24 hours of reperfusion, the region of contraction-band necrosis showed karyolysis and was diffusely infiltrated by neutrophils throughout its extent (Table 1). It was surrounded by a narrow zone of inflammation that appeared enhanced in the interstitial connective tissue around large vessels. Inflammatory infiltration was more diffuse and more uniformly filled the zone of myocyte necrosis than at earlier time intervals but did not extend further into the surrounding region of viable myocytes. The inflammatory infiltrate in the immediate vicinity of blood vessels was no more intense than 3 to 6 hours after the start of reperfusion. Leukocytes, including both neutrophils and cells with single nuclei, were frequently seen within veins in direct contact with the vascular endothelium at all times of reperfusion studied from 1 through 24 hours. Without reperfusion, sections from an infarct produced by 24 hours of permanent coronary artery occlusion showed only limited neutrophil infiltration at the edge of the infarct.

Co-Localization of Neutrophil Emigration and ICAM-1 mRNA Expression

We have previously shown that a border zone surrounds the region of necrosis that is characterized by markedly increased expression of ICAM-1 mRNA in the cardiac myocytes.[22] These cells were apparently exposed to a moderate degree of ischemia insufficient to kill the cells outright, and this border zone of ICAM-1 expression fails to develop in the absence of reperfusion. We have also shown that isolated cardiac myocytes can be killed by adherent neutrophils and that this cytotoxicity is prevented by anti-canine ICAM-1 monoclonal antibody CL18/6.[24,26] Figure 4c shows a border zone containing ICAM-1 mRNA demonstrated by *in situ* hybridization using the antisense riboprobe. The sense riboprobe failed to develop any staining reaction. Figure 4d shows the adjacent serial section stained with H&E, revealing extensive leukocyte infiltration in this region of tissue.

Discussion

In these experiments the circumflex coronary artery was occluded for 1 hour and then released to allow reperfusion of tissue. At the junction between the region of irreversibly injured myocardial cells and the intact cells, an intense local acute inflammatory reaction was seen to develop over the first 3 hours of reperfusion. The border between intact and irreversibly injured myocytes could be identified in longitudinal sections by the presence of uniform contraction-band necrosis in the freshly reperfused infarct. The vessels that were surrounded by the early inflammatory infiltrate were venous structures 10 to 70 μm in width. These vessels were thin walled (approximately 5 μm) but often quite long (up to 500 μm) in sections taken along the axis of the myocytes. In cross sections they appeared ovoid or flattened and tended to run between layers of myocytes. This observation apparently reflects the unique architecture of the venous microvasculature of the mammalian heart. Studies in which beating hearts have been perfused with tracers to allow later study of the microvasculature have shown that cardiac capillaries converge upon short collecting venules in a pattern described by Brown as resembling a turnip root.[37] These drain into sizable veins that run between fascicles or layers of myocytes, within delicate interstitial connective tissue.[37–40] These short, curved venules and interfascicular veins often appear somewhat flattened even in optimally prepared injected specimens, forming a sort of cistern. Some of the vessels surrounded by neutrophils in the immediate subendocardial region may have represented Thebesian venous sinusoids. Recent morphometric studies on the anatomy of the injected coronary venous system of the pig support this description and confirm that the venules and the veins to which they drain are large and flattened in comparison with the postcapillary venules and small veins of skeletal muscle.[41] Thus, the transmigration of neutrophils in response to temporary ischemic injury apparently begins in the collecting venules and interstitial venous cisterns.

The early inflammatory reaction is localized to the border between irreversibly injured and intact myocardium. Of the venous cisterns within intact myocardium, only those within approximately 500 μm of

the edge of the reperfused infarct are surrounded by migrating neutrophils. The neutrophils migrating from vessels within viable myocardium traverse between myocytes, defining a limited region of intact myocardium approximately 500 μm thick, which is intimately exposed to a neutrophilic infiltrate. Thus, the early inflammatory reaction to a reperfused infarct is very limited in its extent relative to the volume of the entire ventricular wall but is quite intense in the vicinity of the cardiac venules and small venous cisterns near the edge of the irreversibly injured zone. Transmigration of neutrophils from blood vessels is known to be dependent upon specific adhesion molecules. ICAM-1 was detected in the endothelium of all cardiac blood vessels, using the CL18/1D8 monoclonal antibody to canine ICAM-1. When the concentration of the primary antibody was reduced stepwise, it was found that the vessels that showed persistent staining were the arteries and veins, including the venules and venous cisterns from which transmigration appeared to originate. P-selectin staining revealed a distribution limited to veins, but again the small collecting venules and venous cisterns appeared to have the greatest concentration of P-selectin of all the vessels in the myocardium. These studies further support the concept that both of these adhesion molecules may have significant roles in allowing transmigration of neutrophils from blood vessels in the heart. In recent studies, Weyrich et al have found that anti-P-selectin reduces injury in a feline model of myocardial ischemia and reperfusion,[14] and other investigators have found that anti-ICAM-1 has cardioprotective effects in myocardial reperfusion models in four species.[9,42–44]

In the period between 1 and 3 hours of reperfusion, neutrophils were observed to accumulate in the extravascular compartment in increasing numbers, and their distribution changed. Margination continued to be observed, and venules and venous cisterns were still surrounded by infiltrating neutrophils. However, although infiltration of the intact myocardial region adjoining the infarct did not expand, the interstices between myocytes gradually became occupied by neutrophils in the periphery of the zone of irreversibly injured myocardium. These morphological studies support the concept that the influx of neutrophils continues to arise predominantly from the venous cisterns but that the migration of neutrophils progresses in the direction of the infarct, gradually occupying more of the interstitium. At 6 hours after reperfusion, the selective clustering of neutrophils around small venous structures is less striking, although neutrophil margination persists. The infiltrate continues to extend further into the necrotic tissue than at 3 hours. After 24 hours of reperfusion, the infarct is almost uniformly occupied by neutrophils. Without reperfusion, sections from a 24-hour infarct showed only limited neutrophilic infiltration at the very edge of the infarct.[17]

Our previous work and that of others has demonstrated accumulation of neutrophils within the previously ischemic area occurring immediately upon reperfusion.[1–4,7,45] The selective localization of these neutrophils correlated with sites of C1q localization and was observable with these techniques for up to 4 hours after reperfusion.[46] In correlation with this, chemotactic activity and the presence of stimulated neutrophils could be demonstrated in postischemic cardiac lymph during the first 4 hours of reperfusion[4]; chemotactic activity was predominantly inhibited by neutralizing antibodies to C5a.[6] The radioactive tracer techniques did not distinguish between neutrophils trapped within the vessels and those transmigrating out of the vessels. Histological examination in the present study suggests that most of the early localization may result from intravascular margination in the venous cisterns. As noted above, conventional histological methods tend to underestimate the extent of infiltration of neutrophils, especially early after reperfusion.[27] The present study demonstrates extensive neutrophil infiltration in the first hour of reperfusion.

The observation of a border zone of intact myocardium into which neutrophil infiltration occurs as early as 1 hour after reperfusion correlates with data from our laboratory suggesting that the initial chemotactic activity arises from lethally injured myocardial cells, from which cardiolipin containing C1q-binding proteins of mitochondrial origin are extruded.[45] Thus, complement activation generating C5a is initiated adjacent to lethally injured cells and would not be expected to extend very far into the normal myocardium. A similar border zone of intact myocardium expresses ICAM-1 mRNA early after reperfusion and expresses ICAM-1 protein between 3 and 6 hours.[22] The presence of neutrophils within this zone during the same time that ICAM-1 expression begins supports the possibility of neutrophil-induced myocyte injury.[23,24,26,47] These observations also suggest that other chemotactic factors may be important in the transmigration of neutrophils and other leukocytes during the course of reperfusion-induced leukocyte infiltration. Infiltration of neutrophils into the infarcted myocardium progresses throughout the 24-hour period observed. Despite the increasingly greater distance of immigration, however, the density of neutrophils does not change a great deal,

suggesting that neutrophil transmigration is ongoing. Neutrophil margination within venous cisterns is observed throughout the 24-hour period. From our previous work, we know that the generation of C5a lasts only 3 to 4 hours after reperfusion.[5,6] We suggest that other chemotactic factors, either general or cell specific, may mediate leukocyte transmigration at later times. Possible candidates for such chemotactic factors are interleukin-8[48] and platelet-activating factor.[49] Our recent work has shown induction of the former in early myocardial ischemia,[50] and there have been reports suggesting a role for platelet-activating factor in myocardial ischemia.[51,52]

The data, when taken as a whole, suggest that the initial localization of neutrophils, which is detectable by radioactive tracer methods, is most intense during the first 4 hours of reperfusion.[4] The early localization is largely accounted for by neutrophil margination in venous cisterns, although robust transmigration is seen within the first hour. Within 3 hours, extensive emigration occurs and corresponds well with the presence of complement-derived chemotactic activity in cardiac lymph.[6] Subsequently, our observations suggest that more limited but ongoing localization and transmigration of neutrophils (and perhaps other leukocytes) persists throughout the 24-hour period. The mechanisms of control of this later process are not yet understood.

Acknowledgments

The authors acknowledge the expert secretarial and clerical assistance given by Michelle Swartout, Lisa Padilla, and Reneé Wicks. The authors also thank Bonnie Hughes and Ann Burke for their technical assistance.

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