Neutrophil Adherence to Isolated Adult Canine Myocytes

Evidence for a CD18-dependent Mechanism

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Abstract

Cardiac myocytes were isolated from adult dogs and incubated with isolated canine neutrophils (PMN). Intercellular adhesion was low and unchanged by stimulation of the PMN with zymosan activated serum or platelet activating factor (PAF) at concentrations that significantly enhance PMN adhesion to protein-coated glass and canine endothelial cell monolayers. Intercellular adhesion was significantly increased only when both myocytes and PMN were stimulated (e.g., myocytes incubated with IL-1, tumor necrosis factor, or phorbol myristate acetate, and PMN were chemotactically stimulated). Inhibitors of protein synthesis diminished the IL-1β-induced effect by > 80%. The IL-1β, PAF-stimulated PMN-myocyte adhesion was associated with substantial H_2O_2 production. Under conditions with low PMN-myocyte adhesion (i.e., IL-1β alone, PAF alone, or no stimulus) H_2O_2 production was generally < 5% of that occurring with high adhesion. An anti-CD18 monoclonal antibody (R15.7) inhibited stimulated PMN-myocyte adhesion by > 95% and reduced H_2O_2 production by > 90%. Control isotype-matched, binding, and nonbinding antibodies were without effect on adherence or H_2O_2 production. The results indicate that cytokine stimulation of adult myocytes induces expression of a ligand involved in CD18-dependent adherence of canine neutrophils. (*J. Clin. Invest.* 1990. 85:1497–1506.) cytokines • hydrogen peroxide • chemotactic factors • monoclonal antibodies • endothelium

Introduction

Neutrophils apparently contribute to the injury of myocardial tissue after occlusion of the coronary artery, particularly under conditions where the ischemic tissue is subsequently reperfused with normally oxygenated blood (1, 2). Neutrophil attachment to vessel walls and emigration into cardiac tissue begins shortly after the initiation of reperfusion (3–8), and various experimental manipulations in animal models designed to remove neutrophils from the circulation (3, 6, 9–12) or to inhibit their functions (13–18) have resulted in measurable reductions in the extent of myocardial damage after ischemia and reperfusion. The mechanisms by which neutrophils effect this tissue damage remain uncertain, though two possibilities have been supported by experimental evidence. The first is that these leukocytes physically obstruct capillaries, thereby limiting reperfusion (19, 20). The second is that secretory products (e.g., oxygen radicals) from the emigrating neutrophils are responsible for cytotoxic effects on myocytes (1, 2, 8). Changes in leukocyte adhesion contribute to both mechanisms. Adherence to capillary endothelium promotes retention of leukocytes within vessels, and is clearly necessary for emigration of neutrophils into extravascular sites (21–23). Additionally, attachment of extravascular neutrophils to tissue cells would provide a small distance over which cytotoxic molecules act, and yield a relatively high concentration of these molecules. Furthermore, it would possibly enhance neutrophil secretory responses (24, 25). While adhesion of neutrophils to extracellular matrix proteins (26–30) and endothelial cells (31–45), and transendothelial migration (42, 43, 46–48) have received considerable attention, including characterization of molecular mechanisms, adhesive interactions of neutrophils with cardiac myocytes have not been evaluated.

In this study we address the hypothesis that inflammation in myocardial tissue promotes the adhesion of neutrophils to cardiac myocytes, and this adhesion augments the secretory activity of neutrophils. Experiments were performed in vitro using isolated myocytes and neutrophils, and defined inflammatory mediators such as cytokines and chemotactic factors. The results demonstrate adhesion of canine neutrophils to canine myocytes in vitro, identify several specific stimuli that activate this adhesion, and provide evidence that production of H_2O_2 is associated with this adhesion. In addition, using a MAb reactive with canine neutrophil CD18, we provide evidence that the stimulated adhesion and H_2O_2 production are CD18 dependent.

Methods

Isolation of cardiac myocytes. Healthy mongrel dogs weighing 10–15 kg were anesthetized using sodium pentobarbital. The heart was removed through the left lateral chest under sterile conditions and immediately placed in ice-cold saline. The aorta was then cannulated using a tubing adapter suitable for the individual heart. The adapter was then connected to a peristaltic pump and retrograde perfusion was initiated at 50–60 ml/min using medium A (Joklik modified minimum essential medium, containing 2 g/liter sodium bicarbonate, 0.1% fatty acid–free BSA and equilibrated with 95% O_2/5% CO_2 before perfusion; Sigma Chemical Co., St. Louis, MO). Perfusion was maintained several minutes until all left ventricle vessels were cleared of blood. The perfusate was then changed to medium B (medium A containing 120 U/ml collagenase type III; Worthington Biochemical Corp., Freehold, NJ) and perfusion continued for 10 min. At this point the heart was removed from the cannulae and 10 g of left ventricle were minced and

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placed in an Erlenmeyer flask containing 50 ml medium B. The flask was equilibrated with 95% O_2/5% CO_2 gas, sealed, and placed in a shaker bath at 90–100 cycles/min at 35°C. After 20 min the supernatant was filtered through one layer of cheesecloth, another 50 ml of medium B was added, and the procedure was repeated five to six times. These cells were allowed to settle at room temperature for ~ 5 min and the overlying solution was removed. The cell pellet was then suspended in medium A and the cells were allowed to settle. This wash was then repeated and viability was measured using trypan blue dye exclusion. Preparations with a viability of > 80% were used in incubation experiments with neutrophils. Cells were then placed on ice and used within one to two days. Viability and adhesion after stimulation were not appreciably altered over this period.

Neutrophil isolation. Canine neutrophils were isolated from citrate anticoagulated venous blood using techniques previously described for the isolation of human neutrophils (43). This yielded a preparation of cells > 95% neutrophils with > 99% viability. These cells were suspended in Dulbecco's PBS and stored at 4°C for up to 4 h. Blood was also obtained on two occasions from a dog with CD18 deficiency (49) (provided by Dr. U. Giger, University of Pennsylvania, Philadelphia).

MAbs. A monoclonal antibody (IgG1) without binding specificity for canine cells was provided as purified IgG from Damon Biotech (Needham Heights, MA) and served as a nonbinding control. The clone producing the MAb LM2/1 (IgG1) (50) was obtained from Dr. T. Springer (The Center for Blood Research, Boston, MA). A new MAb was prepared by immunizing BALB/c mice with canine peritoneal macrophages and subsequently fusing spleen cells with P3X63Ag8.653 myeloma cells according to the protocol of Galfre et al. (51). Supernatants were screened for inhibition of JY cell and SKW3 aggregation (52). Clones positive in both assays were subsequently screened for binding to mouse/human hybrid cells expressing mouse CD11a and human CD18 (53). One clone, R15.7, was selected for further characterization (see Results) as a potential anti–canine CD18 MAb. It was determined to be an IgG1 by Ouchterlony (ICN Biomedicals, Inc., Irvine, CA). Ascites fluid was prepared in pristane-primed BALB/c mice for each of the R15.7 and LM2/1 clones, and IgG was isolated on protein A.

Immunoprecipitation studies. Fresh canine granulocytes (2.2×10^8 cells) were labeled with ^{125}I (10 mCi) by lactoperoxidase and glucosidase as previously described (42). The cells were solubilized in 2 ml of lysis buffer (0.5% NP-40, 10 mg/liter aprotinin, 1 mg/liter pepstatin, 1 mg/liter leupeptin, 1 mM EDTA, 50 μM sodium vanadate, 0.15 M sodium chloride, and 0.15 m Tris, pH 7.6). After removal of cell debris by centrifugation, the lysates (200 μl) were precleared three times with 50 μl glycine-HCl quenched cyanogen bromide–activated Sepharose beads, and then three times with 50 μl of protein A coupled to Sepharose. The lysates (200 μl) were then mixed with the following ascites fluids: 0.5 μl TS1/22, 1 μl OKM-1, and 0.2 μl TS1/18 (clones provided by Dr. T. Springer). The same volume of lysate was also mixed with 2 μl of a polyclonal rabbit anti–human beta subunit and with 5, 10, 25, 50, and 100 μl R15.7 ascites. After a 4-h incubation, 25 μl of protein A bound to Sepharose beads was added with tipping for 2 h. The beads were then washed and boiled in SDS sample buffer (2.5% SDS and 5% mercaptoethanol, wt/vol) for 2 min. SDS-PAGE (7.5% acrylamide) and autoradiography were performed as previously described (42).

Immunofluorescence flow cytometry. Indirect immunofluorescence assessments of the surface expression of binding sites for MAbs LM2/1 and R15.7 were performed using saturating concentrations of the MAbs and FITC-conjugated rabbit anti–mouse IgG (54). Analysis was carried out with a FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, CA). The nonbinding IgG1 served as control. Results are expressed as mean fluorescence channel on a log scale.

Aggregometry and chemotaxis. Homotypic aggregation of canine neutrophils was performed using a technique developed for human neutrophils (55). Phorbol myristate acetate (PMA; Sigma Chemical Co., St. Louis, MO) was used as the stimulus for aggregation, and the neutrophils were incubated with MAb for 5 min at 37°C before addition of the PMA.

Chemotactic migration was determined using the leading front assay with micropore filters as previously described (56). One chemotactic stimulus used in these studies was zymosan-activated canine serum (ZAS).[1] It was prepared by incubating 10 mg of zymosan (Sigma Chemical Co.) in 1 ml fresh canine serum at 37°C for 45 min. The zymosan was removed by centrifugation and the ZAS was heated at 56°C for 30 min. A second chemotactic substance used in these studies was platelet activating factor (PAF; L-α-phosphatidyl-choline,β-acetyl-γ-*O*-alkyl, Sigma Chemical Co.). PAF was initially dissolved in chloroform at a concentration of 200 ng/μl. Before use, the appropriate volume was evaporated to dryness and the PAF suspended at a concentration of 1 μg/ml in PBS containing 1% human serum albumin (HSA; Sigma Chemical Co.). Dilutions used in functional studies were made in PBS without additional HSA.

Canine neutrophil adherence to canine endothelial monolayers and protein-coated glass. Canine jugular vein endothelial cells were obtained by a modification of the method of Ford et al. (57). Jugular veins were everted on glass rods and incubated in collagenase solution (type III, 50 U/ml; Worthington Biochemical Corp.) for 10 min. Cells were collected by centrifugation and suspended in DME containing 4% FCS, 4% bovine calf serum, 50 μg/ml endothelial cell growth factor (Collaborative Research Inc., Lexington, MA), 50 μg/ml heparin, 1 mM sodium pyruvate, and antibiotics. Cells were seeded in Primaria flasks (Becton Dickinson, Lincoln Park, NJ). After 2–4 d of incubation at 37°C in a CO_2 incubator, areas of cells with cobble-stone morphology were collected by scraping, transferred to gelatin-coated flasks, and grown to confluence. Second passage cells were obtained by scraping, seeded onto type I collagen–coated (5 μg/ml) 25-mm round cover glasses, and grown to confluence. Only preparations of cells where representative uniform monolayers exhibited acetylated low density lipoprotein (Dilac-LDL; Biomedical Technologies, Inc., Stoughton, MA) uptake and endothelial (cobble-stone) morphology were used in adhesion assays. Coverslips with attached endothelial monolayers were inserted in adhesion chambers, and adherence of isolated canine neutrophils was determined in the absence of shear stress using a visual assay as previously described (42, 43). In experiments with stimulated endothelial cells, monolayers were exposed to 2 ng/ml LPS (*Escherichia coli;* Sigma Chemical Co.) for 3 h at 37°C, rinsed by dipping the coverslip five times in two changes of PBS before being inserted into the adherence chambers. In experiments with MAbs, neutrophils were exposed to the antibodies at room temperature for 5 min before the cell suspension containing the antibodies was injected into the adherence chambers.

Adhesion of neutrophils to serum-treated glass was determined as previously described (56). Coverglasses were exposed to 3% canine serum in PBS for 2 min, then rinsed in PBS and inserted in the adherence chamber. Adhesion was determined using a visual assay in the absence of shear stress.

Canine neutrophil–myocyte adherence. Isolated canine myocytes were suspended in medium A at a concentration of 50,000/ml. Neutrophils and myocytes were co-incubated in a volume of 0.4 ml at a ratio of 10:1, neutrophils/myocytes, for 30 min at 37°C. The cells were resuspended, a small aliquot was transferred to a microscope slide and covered with a coverglass, and cells were examined under phase contrast or differential interference contrast optics. The percentage of myocytes with > 2 attached neutrophils was determined and the number of neutrophils per myocyte was counted on 200 myocytes per preparation. Samples were coded so that data collection was performed without knowledge of the specific experimental conditions. In experiments with stimulated myocytes, myocytes were incubated with IL-1β and recombinant IL-1β (Genzyme Corp., Boston, MA), tumor necrosis factor (TNFα; Boehringer Ingelheim, Ridgefield, CT), or PMA

1. *Abbreviations used in this paper:* ELAM-1, endothelial leukocyte adhesion molecule-1; HSA, human serum albumin; ICAM-1, intercellular adhesion molecule-1; PAF, platelet activating factor; TNF, tumor necrosis factor; ZAS, zymosan-activated canine serum.

for various times at 37°C before addition of the neutrophils. In experiments with stimulated neutrophils, PAF or ZAS was added immediately before the neutrophil suspension was mixed with the suspension of myocytes. MAbs were added to the neutrophil–myocyte suspension (10 μg/ml) at the beginning and remained with the cells throughout the incubation period.

Evaluation of the effects of protein synthesis inhibitors on the cytokine-induced myocyte–neutrophil adhesion was carried out in the following way: Myocytes were incubated for 3 h at 37°C in the presence of IL-1β (2 U/ml) with and without actinomycin D (5 μg/ml) or cycloheximide (5 μg/ml) (both from Sigma Chemical Co.). Adhesion was assessed as described above using ZAS-stimulated neutrophils. Protein synthesis was assessed by determining the incorporation of [^{3}H]leucine (10 μCi/ml; ICN Biomedicals, Inc., Costa Mesa, CA) added at the same time as IL-1β, using the method of Sen et al. (58).

Evaluation of hydrogen peroxide production. H_2O_2 production was quantitated in 96-well polystyrene microtiter plates by a modification of the method described by Nathan (24, 25). Plates were coated with collagen type I (Collaborative Research Inc.) (150 μl/well of a 0.5 mg/ml solution in PBS) for 30 min at 37°C and washed three times with PBS. The assay reaction mixture (100 μl/well) was 24 μM scopoletin (Sigma Chemical Co.), 5 μg/ml horseradish peroxidase (type II; Sigma Chemical Co.), 1 mM sodium azide, and 5 mM glucose in low phosphate Krebs-Ringer buffer. Unstimulated or stimulated myocytes (4 U/ml IL-1β for 3–4 h at 37°C) were added to appropriate wells (2,000 cells/well). MAbs and PAF were added immediately before the addition of canine neutrophils (6×10^5/well). Experiments were performed in replicates of six to eight each, and fluorescence of scopoletin was determined immediately after addition of neutrophils and at 15-min intervals thereafter in a Titertek Fluoraskan II fluorometer (Flow Laboratories, Inc., McLean, VA) with excitation and emission wavelengths of 355 and 460 nm, respectively. Values for H_2O_2 production were plotted and the areas under the curve determined using a digitizing pad and Sigma-Scan software (Jandel Scientific, Sausalito, CA).

Data presentation. Results are presented as means±1 SD, and n = the number of separate experiments. Statistical assessments were made using analysis of variance and Dunnett's t test or Student's t test.

Results

The rod-shaped appearance of viable isolated myocytes (59) (Fig. 1 *A*) was evident in > 80% of the cells in preparations used in these studies. Co-incubation of neutrophils and myocytes at 37°C for periods up to 1 h resulted in a low incidence of intercellular adhesion with a mean of less than one neutrophil/myocyte. Neutrophils failed to adhere to myocytes with a rounded configuration, cells that typically stained with trypan blue. Exposure of co-incubated neutrophils and myocytes to PMA resulted in a marked increase in adherence (Fig. 2), evident in ~ 1 h. This stimulus resulted in aggregation of neutrophils, the attachment of neutrophils to individual myocytes, and some large clumps of neutrophils containing several myocytes. Since PMA may activate both neutrophils and myocytes, an effort was made to distinguish contributions of each cell type. Stimulation of neutrophils with ZAS (0.1–0.5%) or PAF (5–500 ng/ml) significantly increased adherence to serum-coated glass or monolayers of canine endothelial cells (Table I) (maximum adhesion was reached at 0.2–1.0% concentrations of ZAS and 30–500 ng/ml PAF), induced migration in the leading front assessment of the chemotaxis assay (migration toward serum, 35±5 μm; 1% ZAS, 125±6 μm; 1%

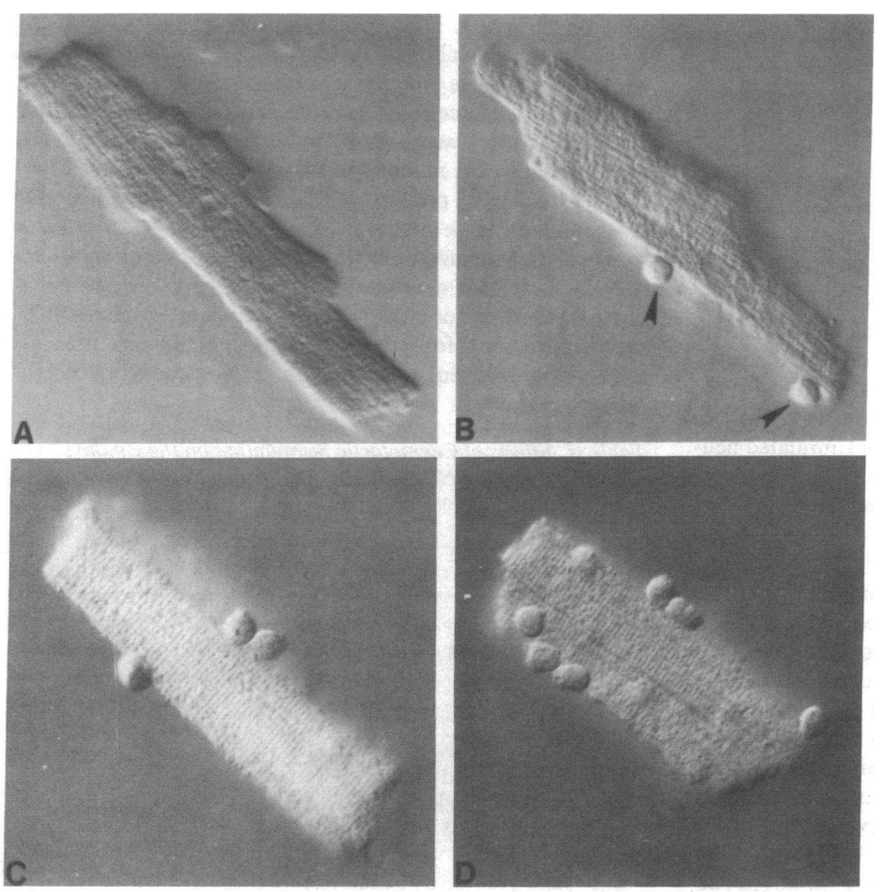

Figure 1. Differential interference contrast images of isolated canine myocytes and attached canine neutrophils. *A*, A myocyte from a control preparation without added stimulants or neutrophils. *B–D*, Myocytes from preparations after stimulation of the myocytes for 3 h with 4 U/ml IL-1, and neutrophils with ZAS (1%). Note the contracted appearance of the myocyte in *D* with numerous attached neutrophils. ×560.

Table I. Effects of Chemotactic Stimulation and MAb on the Adherence of Canine Neutrophils to Serum-treated Glass and Canine Endothelial Monolayers In Vitro

	% Adherence			
Pretreatment of neutrophils*	*n*	Serum, glass	*n*	Endothelium
S, 0.5%	4	25±3	8	33±5
ZAS, 0.1%	4	33±2‡		ND§
ZAS, 0.2%	4	49±3‡	6	65±5*
ZAS, 0.5%	4	50±3‡		ND§
ZAS, 0.2%, R15.7	4	18±2‖	6	33±6‖
ZAS, 0.2%, LM2/1	4	50±3	4	61±4
ZAS, 0.2%, IgG1	4	49±1	3	70±4
1% HSA	5	16±1	4	42±5
PAF, 10 ng/ml	3	96±2‡	4	89±4‡
PAF, 30 ng/ml	4	97±1‡	4	99±1‡
PAF, 200 ng/ml	3	96±1‡	3	98±1‡
PAF, 30 ng/ml, R15.7	4	28±8¶	4	42±5¶
PAF, 200 ng/ml, R15.7	3	32±5¶	3	44±9¶
PAF, 30 ng/ml, LM2/1	4	82±8	4	86±5
PAF, 30 ng/ml, IgG1	4	89±7	3	95±2

* Canine neutrophils were preincubated for 5 min in canine serum (*S*) or ZAS with and without the MAbs indicated (5 μg/ml), and HSA or PAF with and without the MAbs indicated. The solution containing the stimulants and MAbs was injected into the adhesion chamber and adherence determined using a visual assay. Serum-treated glass was prepared by exposing the glass coverslip to 3% canine serum in PBS for 2 min and then washing in PBS. The endothelial cells were exposed to 2 ng/ml LPS for 3 h before being washed and placed in the adhesion chamber. Adhesion of unstimulated neutrophils to unstimulated endothelial monolayers was 9±2%.

‡ $P < 0.01$ compared with control.

§ The effects of this condition were not determined.

‖ $P < 0.01$ compared with 0.2% ZAS stimulation without MAb.

¶ $P < 0.01$ compared with 30 ng/ml PAF stimulation without MAb.

HSA, 32±3 μm; 200 ng/ml PAF, 101±8 μm), and increased the binding of MAbs R15.7 and LM2/1 to the neutrophil surface (Table II). These stimuli failed to increase neutrophil adherence to myocytes over a 1-h observation period (Fig. 3). However, exposure of the myocytes to IL-1β at concentrations as low as 0.5 U/ml for 3 h at 37°C resulted in marked increases in the adhesion of chemotactically stimulated neutrophils (Figs. 1, *B–D* and 3). Stimulation of both the neutrophil and the myocyte appeared to be necessary since unstimulated neutrophils failed to adhere to IL-1β-treated myocytes. The image in Fig. 1 *D* was typical of myocytes with multiple attached neutrophils. Such cells were often distinctly contracted in appearance, and in preparations observed after 1 h co-incubation, many myocytes with attached neutrophils exhibited the rounded configuration of those cells permeable to trypan blue. The IL-1β-induced increase in adhesion appeared to require protein synthesis since addition of inhibitors of protein synthesis markedly reduced adhesion (Fig. 4), and enhanced adherence of neutrophils was not observed when IL-1β was added coincident with the neutrophils and the chemotactic stimulus and incubated for 30 min at 37°C. Increases in adherence were observed after 1 h preincubation of the myocytes with IL-1β (4 U/ml) and reached the peak activity seen in Fig. 3 within 4 h.

Another cytokine (TNFα) promoted adhesion, but in contrast to IL-1β, appeared to be capable of stimulating both cell types since the addition of unstimulated neutrophils to myocyte suspensions containing TNFα resulted in marked increases in adhesion (Fig. 5). Adhesion was evident as early as 1 h after exposure of the myocytes to TNFα, but maximum stimulation was not seen until incubation of the myocytes with TNFα for 3 h before addition of the neutrophils.

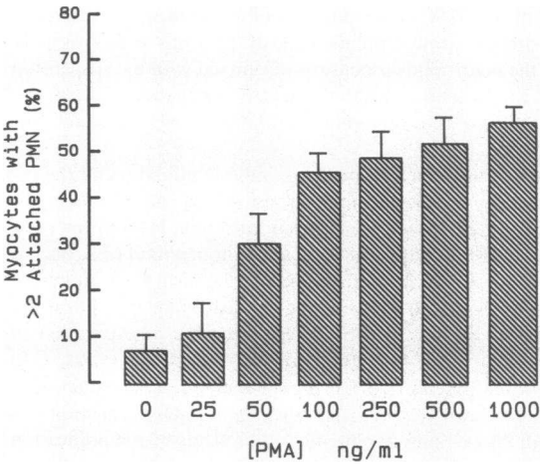

Figure 2. Effect of PMA on adhesion of canine neutrophils to canine myocytes. Neutrophils and myocytes were suspended together in the indicated concentration of PMA at 37°C for 1 h. Adhesion was determined visually and expressed as the percentage of myocytes with more than two attached neutrophils. $n = 6$; $P < 0.01$ for each level of PMA stimulation compared with control.

Hydrogen peroxide production associated with neutrophil–myocyte adherence. H_2O_2 production was evident after incubation of neutrophils and PAF with IL-1β-stimulated myocytes (Table III). As reported by Nathan (24) for human neutrophils on extracellular matrix proteins or endothelial monolayers, there was a considerable lag period before H_2O_2 was detected by the reduction in scopoletin fluorescence. Several controls were performed to assess the requirement for neutrophil–myocyte adhesion. Incubation of neutrophils in type I collagen–coated wells with PAF failed to result in detectable H_2O_2. Canine neutrophils did not adhere to type I collagen–coated plastic or glass surfaces even after prolonged incubations (e.g., 37°C for 2 h, $n = 4$). Exposure of the neutrophils to PAF and unstimulated myocytes did not lead to H_2O_2 pro-

Table II. Effects of Chemotactic Stimulation on the Binding of MAbs to Canine Neutrophils

	Normal			CD18 deficient	
Antibody*	PBS	ZAS	PAF	PBS	ZAS
IgG1	4±1‡	5±2	5±2	4	3
R15.7	85±13	161±22	172±19	5	3
LM2/1	53±9	116±12	121±9	4	4

* Neutrophils from five normal dogs or from a dog with CD18 deficiency were evaluated using flow cytometry. Before labeling with the MAbs and FITC-labeled goat anti-mouse IgG antibody, neutrophils were incubated for 15 min at 37°C in either PBS, 1% ZAS, or 200 ng/ml PAF.

‡ The values given are mean fluorescence channel (±SD) on a log scale.

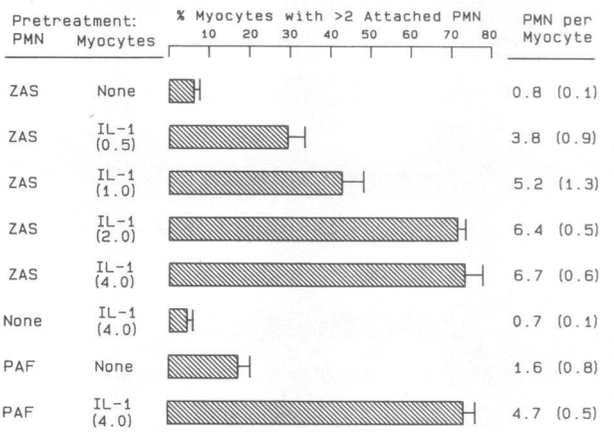

Figure 3. Effect of ZAS, PAF, and IL-1β on the adhesion of canine neutrophils to canine myocytes. Myocytes were incubated at 37°C with and without IL-1β (U/ml) at the indicated concentration before the addition of neutrophils. Both cell types were then suspended together and incubated an additional 30 min at 37°C. Neutrophils were stimulated with ZAS (1%) or PAF (200 ng/ml) 5 min before and during incubation with myocytes. Adhesion was determined visually. PMN per myocyte is expressed as mean (SD), $n = 6$; all values for experiments with both ZAS and IL-1β stimulation are significant, $P < 0.01$, compared with either ZAS alone or IL-1β alone. The values for experiments with both PAF and IL-1β are significant, $P < 0.01$, when compared with either PAF or IL-1β stimulation alone.

duction, and the combination of unstimulated neutrophils with IL-1β-stimulated myocytes was also ineffectual. Finally, IL-1β-stimulated myocytes incubated alone with PAF (200 ng/ml) did not produce detectable H_2O_2.

CD18 dependence of neutrophil–myocyte adhesion and H_2O_2 production. MAb R15.7 binds to CD18 on canine cells as indicated by the following experimental results: (*a*) The clone producing this MAb was initially selected because of its ability to inhibit aggregation of both JY cells and SKW-3 cells, activities consistent with other anti-CD18 MAbs. (*b*) R15.7 bound to mouse–human hybridoma cells expressing hybrid heterodimers of mouse α and human β, but not cells expressing human α and mouse β. This indicates that this MAb recognizes human CD18. (*c*) It failed to bind to neutrophils from humans with CD18 deficiency (not shown) or a dog with

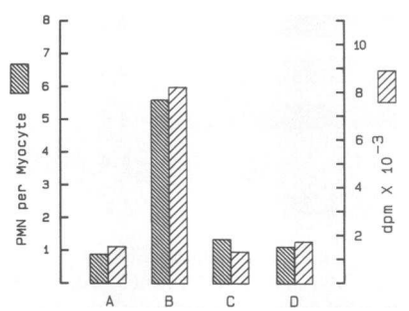

Figure 4. Adhesion of canine neutrophils to canine myocytes: effects of protein synthesis inhibitors. Myocytes were incubated for 3 h at 37°C without or with IL-1β (2 U/ml) and inhibitors (5 μg/ml). Adhesion was determined visually using ZAS-stimulated canine neutrophils, and protein synthesis was assessed by the incorporation of [^{3}H]leucine added at the beginning of the incubation with IL-1β. *A,* Control conditions without IL-1β. *B,* Control levels after stimulation with IL-1β. *C,* IL-1β stimulation in the presence of cycloheximide. *D,* IL-1β stimulation in the presence of actinomycin D. Results represent the mean values from two separate experiments for both adhesion and [^{3}H]leucine incorporation.

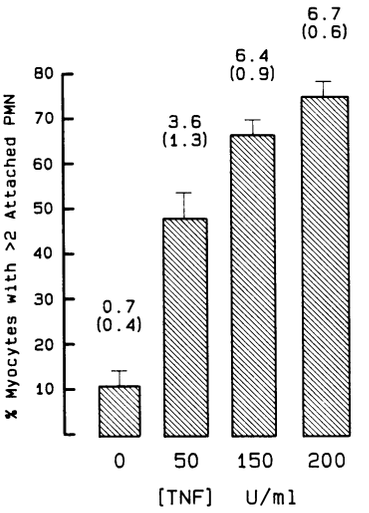

Figure 5. Effect of TNFα on adhesion of canine neutrophils to canine myocytes. Myocytes were incubated with the indicated concentration of TNFα for 3 h at 37°C before the addition of neutrophils to the cell suspension. Incubation was continued for an additional 30 min, and adhesion determined visually. All values for cultures with TNFα were significant, $P < 0.01$, compared with control.

CD18 deficiency (Table II). (*d*) It markedly inhibited homotypic aggregation of human and canine (Fig. 6) neutrophils, a function consistent with the activity of anti-CD18 MAbs on human cells. (*e*) It immunoprecipitated both α and β subunits consistent with leukocyte integrins from lysates of canine neutrophils (Fig. 7) (49).

The adhesion of canine neutrophils to canine myocytes induced by PMA, TNFα, or the combination of chemotactic factor and IL-1β was markedly inhibited by MAb R15.7 (Figs. 8 and 9). Furthermore, the adhesion of canine neutrophils to serum-coated glass and LPS-stimulated canine endothelial

Table III. H_2O_2 Production after Incubation of Canine Neutrophils and Cardiac Myocytes In Vitro

	Experiment number				
Experimental conditions	1	2	3	4	5
Stimulated myocytes + PMN + PAF*	750‡	404	742	700	734
Stimulated myocytes + PMN	5.3§	<5	<5	11.9	67.4
Stimulated myocytes + PAF	<5	ND	<5	<5	<5
Unstimulated myocytes + PMN + PAF	ND‖	75.7	<5	<5	<5
Unstimulated myocytes + PMN	ND	<5	<5	<5	<5

* Myocytes were stimulated for 3 h with IL-1β, 4 U/ml, and then mixed with neutrophils and PAF (200 ng/ml) in type I collagen-coated microtiter plate wells. The reduction in scopoletin fluorescence was used to determine the production of H_2O_2.

‡ Expressed as picomoles/well of H_2O_2 produced over a 100-min observation period beginning after the initial lag phase. The lag phase in minutes for each of these experiments was 75, 150, 120, 30, and 25, respectively.

§ Expressed as percent of the H_2O_2 produced by stimulated myocytes + PMN + PAF, determined using area under the curve of values plotted at 15-min intervals. Values given as < 5 reflect H_2O_2 production too low to accurately measure.

‖ The effects of this condition were not determined.

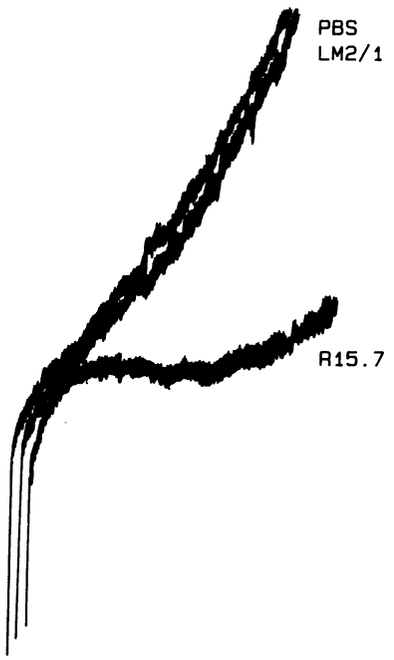

Figure 6. Homotypic aggregation of canine neutrophils: effects of MAbs R15.7 and LM2/1. Isolated canine neutrophils were suspended in PBS and stimulated with PMA (200 ng/ml), and aggregation was followed for 5 min. Representative tracings show inhibitory activity of R15.7.

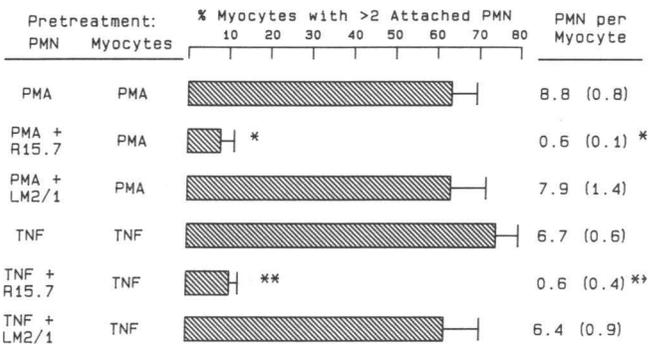

Figure 8. Effects of MAbs on the adhesion of canine neutrophils to canine myocytes. Myocytes were incubated with PMA (100 ng/ml) for 1 h at 37°C or with TNFα (100 U/ml) for 3 h at 37°C. Neutrophils were incubated with the stimuli and MAbs indicated for 5 min before addition to the myocytes suspension. PMA, TNFα, and MAbs were retained with the cell suspensions, incubation was continued at 37°C for an additional 30 min, and adhesion was determined visually. *$P < 0.001$ compared with control without MAb; **$P < 0.001$ compared with control without MAb.

monolayers was increased by ZAS and PAF. This increase was blocked by MAb R15.7 (IgG1) but not by LM2/1 or IgG1 (Table I). Adhesion of neutrophils to unstimulated endothelial monolayers was increased by ZAS (control with no stimulation, 9±2% adherence; with 0.5% ZAS for 10 min, 18±4% adherence, $P < 0.05$, $n = 4$). This increase was blocked by antibody R15.7 (with 0.5% ZAS for 10 min in the presence of 5 μg/ml R15.7, 7±4% adherence, $P < 0.05$, $n = 3$) but not LM2/1 (17±2% adherence, $n = 3$).

A second MAb, LM2/1 (IgG1), served as a control. This antibody, previously shown to recognize CD11b on human cells, binds to the surface of canine neutrophils but fails to block adhesive functions that in human cells are mediated by Mac-1 (CD11b/CD18). For example, LM2/1 failed to inhibit homotypic aggregation of canine neutrophils (Fig. 6) and adhesion of canine neutrophils to serum-coated glass (Table I). This MAb failed to inhibit adhesion of canine neutrophils to canine myocytes (Figs. 8 and 9) and LPS-stimulated canine endothelial monolayers. A nonbinding control IgG1 also failed to inhibit adhesion in these settings (Table I and Fig. 9).

As shown in Table IV, H_2O_2 production stimulated by IL-1β and PAF was reduced to low levels by addition of R15.7 (5 μg/ml). Control antibodies, IgG1 (5 μg/ml), and LM2/1 (5 μg/ml) did not inhibit H_2O_2 production.

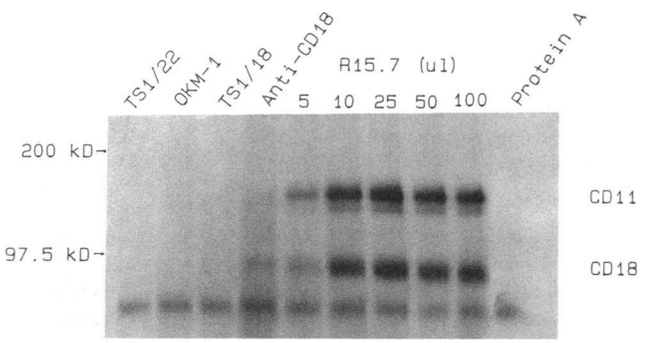

Figure 7. Immunoprecipitation of canine neutrophil lysates with antibodies TS1/22 (anti-human CD11a), OKM1 (anti-human CD11b), TS1/18 (anti-human CD18), anti-CD18 (rabbit polyclonal anti-human CD18), and R15.7 (anti-CD18). TS1/22 and OKM1 fail to bind to canine neutrophils or to immunoprecipitate antigen from canine cells. TS1/18 and the polyclonal anti-CD18 react weakly with canine cells. R15.7 reacts strongly with canine and human cells and immunoprecipitates at least two α-chains (indicated as CD11 on this figure), a β-chain from canine neutrophils, and three α-chains and a β-chain from human neutrophils (not shown).

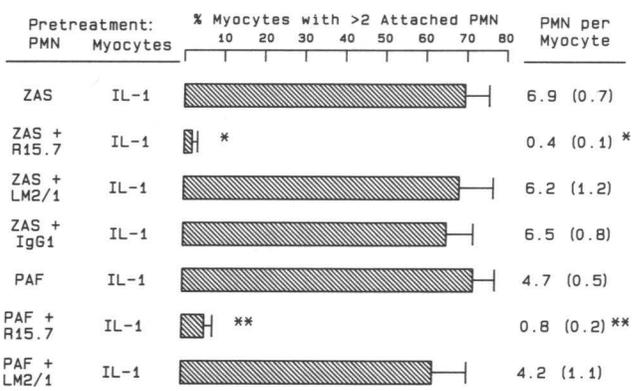

Figure 9. Effects of monoclonal antibodies on the adhesion of canine neutrophils to canine myocytes. Myocytes were incubated with IL-1β (4 U/ml) for 3 h at 37°C. Neutrophils were incubated with the stimuli (1% ZAS or 200 ng/ml PAF) and MAbs indicated (R15.7, anti-CD18; LM2/1, anti-CD11a; IgG1 nonbinding control) for 5 min before addition to the myocyte suspension. The stimuli and MAbs were retained with the cell suspensions, incubation was continued at 37°C for an additional 30 min, and adhesion was determined visually. *$P < 0.001$ compared with control without MAb; **$P < 0.001$, compared with control without MAb.

Table IV. Effects of MAb R15.7 on H_2O_2 Production by Canine Neutrophils Incubated with Canine Myocytes

Experimental conditions	Experiment number 1	2	3	4	5	Mean±SD
R15.7, 5 μg/ml*	7.2‡	15.8	7.5	10.9	4.6	9.2±4.3
LM2/1, 5 μg/ml	ND§	97.5	118.1	99.9	ND	105.2±11.3
IgG1, 5 μg/ml	ND	132.0	99.7	99.1	100.2	107.7±16.2

* Myocytes were stimulated for 3 h with IL-1β, 4 U/ml, and then mixed with neutrophils, MAb as indicated, and PAF (200 ng/ml) in type I collagen-coated microtiter plate wells. The reduction is scopoletin fluorescence was used to determine the production of H_2O_2.
‡ Expressed as percent of the H_2O_2 produced by stimulated myocytes + PMN + PAF, determined using area under the curve of values plotted at 15-min intervals.
§ The effects of this condition were not determined.

Discussion

Adhesion of human neutrophils to human endothelial cells clearly involves CD18 (33, 35, 36, 41–43, 60, 61). Chemotactic stimulation of the neutrophils significantly increases adherence, and this increase is largely CD18 dependent, since anti-CD18 MAbs such as TS1/18 and 60.3 produce > 90% inhibition, and adherence of neutrophils from patients with CD18 deficiency is not increased by chemotactic stimulation. Stimulation of the endothelial cells with LPS, IL-1β, or TNFα markedly increases adhesion of unstimulated neutrophils. This increase is partially dependent on ligand(s) for CD18 heterodimers as shown by the finding that the anti-CD18 antibodies produce ~ 50% inhibition, and adhesion of CD18-deficient cells reaches only approximately half that of normal neutrophils adhering to cytokine-stimulated endothelial cells.

Canine neutrophils apparently utilize CD18 in their adherence to canine endothelial cells as shown by the results in this report. A MAb (R15.7) shown to recognize canine CD18 markedly inhibited the augmented adhesion induced by chemotactic stimulation. In addition, canine neutrophils also appear to utilize CD18 in adherence to cardiac myocytes. Stimulation of both cell types appears to be necessary and accounts for the results obtained with PMA and TNFα. Studies of human cells have shown that these agents activate both neutrophils and endothelial cells to promote intercellular adherence (35, 41). IL-1β, PAF, and ZAS used alone are apparently unable to stimulate both canine neutrophils and myocytes, since combinations of IL-1β and chemotactic stimulation were required for adherence. These results are also consistent with the response of human neutrophils and endothelial cells (42). Thus the canine neutrophil integrin involved appears to require activation since adhesion of unstimulated neutrophils to cytokine-stimulated myocytes was not increased over that of unstimulated neutrophils to unstimulated myocytes. The current experiments do not distinguish between a requirement for mobilization of CD18 from internal pools (55, 62–64) (a phenomenon that may explain the increased binding of MAbs R15.7 and LM2/1 seen in Table II), and the activation or clustering of existing surface integrin molecules, phenomena reported to occur on human neutrophils (65–69) but as yet unknown on canine cells.

The putative ligand on the myocyte involved in the attachment of neutrophils does not seem to be constitutively expressed since stimulation (e.g., with IL-1β) and protein synthesis are required for increased adhesion. While the identity of this ligand is unknown at present, two prominent human endothelial ligands for neutrophil adhesion, intercellular adhesion molecule-1 (ICAM-1) (39, 42, 43, 52) and endothelial leukocyte adhesion molecule-1 (ELAM-1) (39, 44), may have canine homologues. The CD18 dependence of the canine neutrophil–myocyte adhesion appears to exclude a role for an ELAM-1-like ligand, since adhesion of neutrophils to ELAM-1 does not appear to require CD18 heterodimers (70). ICAM-1 is a candidate since adhesion of neutrophils to this ligand is CD18 dependent (42, 43), and ICAM-1 is expressed on diverse cell types after cytokine stimulation (71). However, since CD18 heterodimers have been shown to form adhesive interactions with several different molecules (27, 72, 73), the adhesive ligand on stimulated myocytes may be distinct from those on endothelial cells.

The co-incubation of PAF-stimulated neutrophils with IL-1β-stimulated myocytes is accompanied by substantial production of H_2O_2. This phenomenon appears to be consistent with the adherence-dependent H_2O_2 production obtained with human neutrophils contacting extracellular matrix proteins or human endothelial cells (24, 25). The oxidative burst in our experiments with canine cells requires neutrophil–myocyte adhesion. This conclusion is supported by the finding that H_2O_2 production did not occur in the absence of adhesion: (*a*) Neutrophils stimulated with PAF on type I collagen–coated plastic, a nonadhesive surface, did not produce H_2O_2. (*b*) Myocytes stimulated with IL-1β and PAF in the absence of neutrophils did not produce H_2O_2. (*c*) Neither unstimulated neutrophils on IL-1β-stimulated myocytes nor stimulated neutrophils on unstimulated myocytes produced H_2O_2. (*d*) H_2O_2 production was inhibited in the presence of a MAb (R15.7) that blocked adhesion, but was not altered by the presence of antibodies (IgG1 and LM2/1) that did not alter adhesion. Thus it appears that chemotactically stimulated H_2O_2 production by canine cells, like that by human cells, is markedly potentiated by adhesion, and the CD18-dependent adhesion of neutrophils to myocytes can participate in this potentiation.

The occurrence and possible roles of neutrophil–myocyte adhesion and H_2O_2 generation in inflammatory injury (e.g., ischemia/reperfusion) to myocardial tissue remain to be defined. The plausibility of some contribution to tissue injury is supported by numerous studies in animal models of cardiac ischemia/reperfusion, and recent experiments in vitro. Antioxidants (e.g., superoxide dismutase and catalase) clearly reduce the area of tissue damage (74–76), though the specific sites of action have not been defined. Also, antiadhesive therapy, the use of the MAb 904 (anti-CD11b/CD18) (15, 77), reduced the area of tissue damage in a canine model, though the specific adhesive events blocked by this antibody in vivo have not been defined. Lymph draining ischemic and reperfused canine cardiac tissue has been found capable of activating canine neutrophil adhesion to canine endothelial cells in vitro (78), and in recent studies (unpublished data) we found that dilutions of postischemic canine cardiac lymph can, over a 3-h period, induce myocytes to become adhesive for ZAS-stimulated neutrophils. In addition, isolated cardiac myocytes are clearly susceptible to damage by H_2O_2 (79). Such observations support the possibility that the neutrophil–myocyte adhesion and H_2O_2 production observed in the present report may play significant roles in myocardial inflammation.

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