Severity of myocardial injury following ischemia-reperfusion is increased in a mouse model of allergic asthma

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Submitted 22 December 2005; accepted in final form 8 August 2006

INFLAMMATION PLAYS a key role in the development and progression of cardiovascular diseases. Blood levels of nonspecific markers of systemic inflammation, such as C-reactive protein (2, 3, 24) and serum levels of myeloperoxidase (MPO), are indicative of the risk for adverse cardiovascular events (4, 5). In addition, neutrophil infiltration is a defining feature of ischemic cardiac diseases (15). Reperfusion injury following revascularization procedures is a major cause of myocardial tissue damage, and, accordingly, reperfusion elevates proinflammatory cytokines and infiltration of neutrophils in the tissue (11, 21, 32). IL-1β and TNF-α have direct effects on cardiomyocytes (6, 30), and TNF-α and IL-6 prime inflammatory cells, increasing their response to soluble proinflammatory mediators such as N-formyl peptides, C5a, and platelet-activating factors (15). Infiltrated neutrophils cause tissue destruction by release of elastases, proteases, and superoxide radicals (15, 27, 28).

Inflammation, both at the airway and systemic levels, is also a key characteristic in asthma pathogenesis (16, 26), and epidemiological evidence indicates cardiovascular complications are increased in long-standing cases of asthma (10, 20, 31). Since drugs used for the treatment of asthma, such as β-agonists, theophyllin, and steroids, have significant cardiovascular side effects, the cardiovascular complications in asthma are often attributed to the respiratory drug therapy. However, evidence indicates that inflammation induced by airborne agents may contribute to these effects. Eosinophils are the primary infiltrating cell type in asthmatic airways, but neutrophils are also observed (12, 19). In addition, serum levels of cytokines that act to prime inflammatory cells, such as TNF-α, IL-4, IL-5, and IL-6, have been shown to be elevated in asthmatic patients (22).

Using a rabbit model of allergy and asthma, we (7) recently demonstrated that myocardial ischemia-reperfusion (I/R) injury (IRI) is more pronounced following exposure to aerosolized allergen, suggesting that asthma and its associated systemic inflammation directly impact cardiovascular injury associated with I/R. The purpose of this study was to test the hypothesis that the effects of pulmonary inflammation on myocardial I/R is neither antigen nor species specific and to define the potential mechanisms for the increased injury observed with airborne allergen exposure. In the present study, a murine model of allergic asthma was used to determine the systemic aspects of the allergic response that could explain the enhancement in IRI.

MATERIALS AND METHODS

Animal use. All animal procedures conformed to the standards in the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals and were approved by the East Carolina University Institutional Committee on the Care and Use of Laboratory Animals.

Allergic sensitization and challenge. Male mice (BALB/C mice, 6–8 wk old) were sensitized as described previously (17) and summarized in Table 1. Briefly, the animals were sensitized by intraperitoneal injection of ragweed (RW) or normal saline (NS) and challenged by intratracheal instillation of RW or NS. Effective allergenic sensitization and challenge were confirmed by hyperresponsiveness to aerosolized methacholine (MCh) and bronchoalveolar lavage. In vivo myocardial ischemia-reperfusion injury was induced by ligation of the left anterior descending artery for 20 min, followed by reperfusion for 2 h. The infarct size (% risk area) and neutrophil density in the myocardial area at risk were significantly higher in the RW/RW group than in the control groups. The tissue neutrophil count correlated with the infarct size but did not correlate with blood neutrophil counts. Furthermore, in the RW/RW group, circulating granulocytes showed an enhanced expression of CD11b and P-selectin glycoprotein ligand-1, enhanced stimulated release of myeloperoxidase, and enhanced expression of P-selectin in the coronary vasculature. These results indicate that allergic responses in the Airways enhance expression of attachment molecules in coro- nary vasculature and activate circulating neutrophils, resulting in recruitment of highly activated neutrophils to the infarct zone during an acute ischemia-reperfusion event, thereby enhancing tissue destruction.

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Pulmonary function testing. Effective sensitization and challenge were confirmed by responsiveness to aerosolized methacholine (MCh)
and bronchoalveolar lavage (BAL) in a subset of animals chosen at random. These animals were excluded from the I/R protocol due to possible confounding effects of MCh challenge on IRI.

Twenty-four hours after the last intratracheal challenge, conscious animals were placed in the plethysmograph chamber (Buxco Electronics, Sharon, CT), and activity was allowed to stabilize for 10 min. Enhanced pause (P_{e,nh}) was measured as an indirect assessment of airway resistance. Following equilibration, animals were exposed to nebulized saline for 4 min, and P_{e,nh} was recorded for 5 min to establish a baseline. MCh was dissolved in normal saline at concentrations of 0.75–48 mg/ml. Animals were exposed to increasing doses of nebulized MCh for 4 min. P_{e,nh} was monitored continuously for 5 min following each dose of MCh.

**BAL cell counts.** Twenty-four hours after the last intratracheal challenge, animals were euthanized with pentobarbital sodium (120 mg/kg ip), and lungs were lavaged with 0.5 ml of PBS. Total cell counts were determined by using a Coulter counter (Beckman Coulter Instruments; Miami, FL). Differential cell counts were obtained by counting a minimum of 200 cells on cytocentrifuge preparations stained with Diff-Quick.

**Peripheral blood leukocyte count.** Blood was collected from the tail vein in heparinized vials from each animal before induction of ischemia. Following red blood cell lysis, total white blood cell counts were determined by using a Coulter counter. Smears were prepared and stained with Diff-Quick for differential cell counts. Differential counts were obtained by counting 200 cells per slide and two slides per animal.

**IRI.** IRI was induced essentially as described by Ahn et al. (1) and Jones et al. (14) 24 h after the last intratracheal challenge. Briefly, animals were anesthetized with pentobarbital sodium (60 mg/kg ip), and supplemental pentobarbital sodium was administered at 30 mg/kg as needed. A midline tracheotomy was performed. The animals were intubated with PE-90 tubing, connected to a rodent ventilator (Harvard 683), and mechanically ventilated with 100% oxygen at 120 strokes/min and a tidal volume of 70 ml/kg (1.2 ml/stroke). After 10 min of equilibration, the thorax was opened by a left parasternal incision, and the pericardium was carefully separated. With a reversable snare, the left anterior descending coronary artery (LAD) was ligated ~4 mm distal to its origin between the conus arteriosus and the left auricle. Effective occlusion was confirmed by visual inspection of cyanosis of the myocardial tissue distal to the ligature. After 20 min of occlusion, the snare was released and the heart was reperfused for 2 h. The time was chosen based on the empirical observation that 20 min of occlusion resulted in a reproducible infarct size in control animals and allowed for further infarction without increased mortality. Twenty-minute ligation of the LAD has been reported previously in the murine model (14). Body temperature was maintained at 37°C using an electric warming blanket. Desiccation was minimized by reapproximating the chest walls and covering the thoracotomy with Parafilm.

**Determination of infarct size.** The location of the ligature 4 mm distal to the conus was chosen based on preliminary experiments indicating that occlusion at this level placed ~50% of the myocardium at risk of ischemic injury. The area at risk data, reported as a percentage of the total left ventricle (LV), include correction for the positioning of the ligature and are provided only as an estimate of consistency in the production of an experimental infarction. Measured infarction data are reported as a fraction of the risk area. At the completion of the I/R protocol, the coronary ligature was retied, a cannula was inserted into the aorta, and the risk area was identified by exclusion following retrograde aortic perfusion using a 1% solution of Evans blue dye.

After Evans blue staining, the hearts were excised and cut into serial sections, ~1 mm thick, distal to the ligature. All sections were incubated in 1% solution of 2,3,5-triphenyltetrazolium chloride for 10 min to demarcate the infarcted from the noninfarcted tissue. Both apical and basal surfaces of each section were photographed using a digital camera, and the LV area, area at risk, and area of infarction in each image were determined using NIH image software (ImageJ, version 1.34s). Values from each section were averaged to obtain a value for each animal. Average values for each animal were used for statistical summary and comparisons of groups.

**Myocardial histology.** Histology was performed on the same tissue sections used for infarct measurements. Following triphenyltetrazolium chloride staining and digital photography, myocardial sections were fixed in 10% formalin, embedded in paraffin blocks, and cut at 5-μm sections. Sections were stained with Gill’s hematoxylin and eosin and examined by light microscopy under high magnification (×40). The number of infiltrated neutrophils in each high-power field was counted and normalized to the area of the field, based on the microscope specifications. Neutrophil counts were made on five sections per mouse heart and on three fields per section.

**Myeloperoxidase assay.** Whole blood was collected 24 h after the last airway challenge and pooled. Neutrophils were enriched by sedimentation through a discontinuous Percoll gradient (1.10, 1.095, and 1.085 g/ml Percoll in PBS). Total cell count in the isolate was done by using a Coulter counter (Beckman), and the neutrophil percentage in the isolate was determined by examining cytospin preparations from the isolate stained with Diff-Quick. Isolated neutrophils were adjusted to 5 × 10^5 cells/ml of media, based on the total cell count and percent purity of neutrophils in the isolates. The assay was done in four replicates from each sample. Isolated neutrophils were incubated in a water bath at 37°C with cytochalasin B (0.01 μg/μl) and N-formyl-Met-Leu-Phe (MLP, 1 μM/μl) for 10 min, and a substrate for MPO (Diaminobenzidine, D-9143; Sigma; St. Louis, MO) was added to the supernatant. A reading for absorbance at 450 nm was done after 5 min. The absorbance was reported as MPO units.

**Granulocyte surface markers.** Flow cytometry was performed to measure relative expression of P-selectin glycoprotein ligand-1 (PSGL-1), L-selectin, and CD11b. Whole blood was pooled from a minimum of five animals, and the red blood cells were lysed with ammonium chloride buffer (pH 7.4). After being blocked with 10% rat serum, 200 μl of white blood cell suspension in PBS were incubated with L-selectin-FITC, PSGL-1-phycocerythrin, CD11b-phycocerythrin-Cy7 antibodies, or directly conjugated isotype control antibodies (Biodign). The cells were washed and resuspended in PBS. Granulocytes, lymphocytes, and monocytes were identified by forward and side-scatter properties. Data were acquired by using a Becton Dickinson FACScan flow cytometer and analyzed by using Cell Quest software.

**Immunohistochemistry.** Formalin (4%)-fixed sections of the I/R-induced myocardium were used for immunohistochemistry to detect expression of P-selectin in the coronary vasculature. Sections were cut at 5 μm thickness, and slides were boiled in 0.6 M sodium citrate buffer (pH 6.0) for 20 min for antigen retrieval. Sections were then blocked with donkey serum for 2 h at room temperature, followed by incubation with polyclonal goat anti-P-selectin antibody [dilution 1:100 in PBS + Tween 20 (PBST), Santa Cruz]. Donkey anti-goat IgG-TR (1:500 dilution in PBST, Santa Cruz) was used for detection, and slides were visualized by using fluorescence microscopy. Two sections (from 5 to 6 animals/group) were analyzed for expression of...
with enhanced IRI in allergic mice. However, the possibility that increased tissue neutrophil infiltration was associated with allergen challenge protocol induced hyperresponsiveness to MCh and pulmonary and systemic inflammation.

Myocardial infarct size following I/R is increased in ragweed-sensitized and -challenged mice. The LV area at risk after I/R injury was normalized to total LV area in each mouse. There was no significant difference in the area at risk across all groups (Fig. 2B), indicating that the placement of the ligature was consistent across the groups. Following I/R, the RW/RW group exhibited a greater infarct size compared with that in the control groups (Fig. 2A; RW/RW, 50.4 ± 6.4%; RW/NS, 31.3 ± 4.1%; and NS/NS, 27.7 ± 3.6%; means ± SE; n = 8/group; P < 0.001). These results confirmed that the allergen challenge protocol induced hyperresponsiveness to MCh and pulmonary and systemic inflammation.

Myocardial tissue neutrophil infiltration associated with infarct size. Neutrophil infiltration into the myocardial area at risk was quantified. Ragweed-sensitized and -challenged animals exhibited a higher number of neutrophils in the myocardial area at risk compared with that in control animals (Fig. 3; RW/RW, 559 ± 32; RW/NS, 459 ± 35; and NS/NS, 462 ± 20 cells/mm²; means ± SE; n = 8/group; P < 0.05). Therefore, increased tissue neutrophil infiltration was associated with enhanced IRI in allergic mice. However, the possibility that increased neutrophil influx was secondary to enhanced tissue destruction could not be excluded.

A correlation analysis was performed to evaluate the association between tissue neutrophil count and infarct sizes. Myocardial infarct size in all groups showed a significant positive correlation to the tissue neutrophil count (Fig. 4A; RW/RW, R = 0.82; RW/NS, R = 0.85; and NS/NS, R = 0.83; P < 0.001).

Fig. 1. Induction of airway hyperresponsiveness to methacholine (MCh) and local and systemic inflammation by ragweed. A: Airway reactivity assessed by whole body plethysmography 24 h after intratracheal administration of ragweed (8 μg) or normal saline. Mice were exposed to nebulized MCh in the range of 0.75–48 mg/ml. RW/RW, mice sensitized and challenged with ragweed (n = 7); RW/NS, mice sensitized with ragweed and challenged with normal saline (n = 6); NS/NS, mice sensitized and challenged with normal saline (n = 5); *P < 0.05. Penh, enhanced pause. B: Differential leukocyte counts in the bronchoalveolar lavage (BAL) fluid of RW/RW (n = 6), RW/NS (n = 5) and NS/NS (n = 5) mice. Lavage was done 24 h after the last airway challenge; *P < 0.05. L, lymphocytes; E, eosinophils; N, neutrophils; M, macrophages. C: Differential leukocyte counts in the peripheral blood of mice. Blood was collected from the tail vein before induction of ischemia. TLC, total leukocyte count; B, basophils. *P < 0.001, significant difference between the groups in the corresponding cell types (n = 8 in each group).
There was no statistically significant correlation between peripheral blood neutrophil count and myocardial infarct size (Fig. 4B; RW/RW, R = 0.18; RW/NS, R = 0.18; and NS/NS, R = 0.10; P > 0.05, power = 0.91), and peripheral blood neutrophil count also had no significant correlation to the tissue neutrophil count (Fig. 4C; RW/RW, R = 0.09; RW/NS, R = 0.23; and NS/NS, R = 0.24; P > 0.05; power = 0.97). Together, these data indicate that the enhanced neutrophil infiltration in the myocardial area at risk in the RW/RW group was not driven by the increased neutrophil count in the peripheral blood. Alternative explanations for increased tissue neutrophils in the ischemic region are enhanced neutrophil recruitment factors on the vascular interface.

**Ragweed sensitization and challenge increases fMLP-stimulated MPO release from circulating neutrophils.** MPO release from blood neutrophils was measured as an indicator of the activation state of circulating cells. MPO release was higher in the RW/RW group compared with that in the control groups (Fig. 5; RW/RW, 0.63 ± 0.03; RW/NS, 0.50 ± 0; and NS/NS, 0.50 ± 0 MPO units, P < 0.05). The results indicate that ragweed sensitization and challenge primes circulating neutrophils to produce a larger oxidative burst if stimulated, which could enhance myocardial damage following I/R.

**Circulating granulocytes from ragweed-sensitized and -challenged mice show an enhanced activation state.** Expression of PSGL-1 and CD11b on peripheral granulocytes was determined by flow cytometry on pooled whole blood from RW/RW mice and sham-operated controls (Fig. 6 and Table 2). Data are representative of four experiments on pooled blood samples from five animals in each group. Histogram represents the mean fluorescence intensity units (MFU) of the gated granulocyte population. Right shift of the MFU in the RW/RW group suggests that PSGL-1 and CD11b expression in circulating granulocytes in this group are enhanced compared with that in the sham-operated controls. There was no difference in the expression of L-selectin. Increased expression of PSGL-1 and CD11b is consistent with enhanced neutrophil adhesion in microvasculature and infiltration into ischemic myocardium.

**Endothelial P-selectin expression is enhanced in coronary vasculature in the ragweed-sensitized and -challenged mice.** P-selectin expression in the coronary vasculature in the myocardial area at risk was examined by immunofluorescence microscopy. Ragweed sensitization and challenge enhanced expression of P-selectin on coronary endothelium (Fig. 7, A and B; average integrated density normalized to vessel perimeter: RW/RW, 1068.5 ± 120.5; RW/NS, 205.5 ± 105.6; and NS/NS, 252.7 ± 104.9 pixels; means ± SE; P < 0.001, compared with RW/NS and NS/NS). In all three groups, P-selectin expression could not be detected in myocardial tissue isolated from animals that did not undergo the I/R procedure. Upregulation of P-selectin is consistent with enhanced neutrophil infiltration of the myocardium in the infarct.

**DISCUSSION**

Aeroallergen challenge in allergic mice increased myocardial tissue infiltration of neutrophils and myocardial infarct size following acute I/R. A strong correlation between tissue neutrophil density and infarct size indicated that neutrophil influx into the myocardium might be responsible for enhanced myo-

**Fig. 2. Effects of ragweed challenge on ischemia-reperfusion injury.** A: myocardial infarct size normalized to the area at risk (AAR; n = 8 in each group; *P < 0.05). B: left ventricular (LV) AAR of the different groups after ischemia-reperfusion injury normalized to total LV area. There was no significant difference in the AAR across the groups (n = 8 in each group).

**Fig. 3. Myocardial tissue neutrophil counts in the AAR.** Paraffin sections were cut from formalin-fixed tissue and stained with hematoxylin and eosin. Sections were examined under light microscope, and neutrophils were counted under ×40 magnification. Neutrophil counts were made in 5 sections from each heart and in 3 different fields in each section. The tissue area in each field was calculated based on the microscope specifications, and neutrophil count is shown (in cells/mm² of the AAR; n = 8 in each group; *P < 0.05).
cardial damage. An increase in blood neutrophils could not explain the tissue neutrophil infiltration. Enhanced expression of adhesion molecules on both circulating neutrophils and the coronary endothelial cells indicated that activation of both cell types contributed to the enhanced neutrophil extravasation into the tissues. Taken together, the findings indicate that aeroallergen challenge in allergic subjects enhance polymorphonuclear neutrophils (PMN) infiltration into ischemic myocardial tissues through concerted activation of circulating neutrophils, coronary endothelium, and ischemic myocardial tissue.

Recent studies (18, 23, 33, 35) have revealed a strong association between ischemic cardiovascular events and particulate air pollution. As reviewed by Kaiser (18), epidemiological studies have shown that increased levels of ambient airborne particulate matter are associated with increased cardiovascular morbidity and mortality. These findings support the idea that exposure to environmental stimuli which induce pulmonary inflammation predisposes individuals to more severe cardiovascular injury in response to an ischemic event.

The results of the current study are consistent with, and provide potential mechanistic explanations for, epidemiological findings. Induction of allergic lung inflammation was associated with both an increase in the density of neutrophils in the myocardial infarct as well as an increase in the activation state of the circulating neutrophils. Neutrophil MPO is one of the key enzymes responsible for the production of reactive oxidative species and, hence, tissue destruction. MPO is stored in granules within the cytoplasm and is released on activation of neutrophil surface receptors (34). The finding that circulating neutrophils in ragweed-sensitized and -challenged animals exhibited increased MPO release from circulating neutrophils (RW/RW, 0.63 ± 0.03; RW/NS, 0.5 ± 0; and NS/NS, 0.5 ± 0 MPO units, *P < 0.05).

Fig. 4. Regression analyses of myocardial infarct size and inflammation. A: significant positive correlation between tissue neutrophil count and myocardial infarct size. (RW/RW, R = 0.82; RW/NS, R = 0.83; and NS/NS, R = 0.83; *P < 0.001). B: no significant correlation between peripheral blood neutrophil count and tissue neutrophil count, thereby suggesting that alteration of local tissue parameters, such as increased expression of neutrophil chemokines or adhesion molecules or activation of a subset of circulating neutrophils, may be responsible for enhanced neutrophil recruitment to the tissue (RW/RW, R = 0.09; RW/NS, R = 0.23; and NS/NS, R = 0.24; *P > 0.05).
microcirculation even in nonischemic conditions. In the study by Ritter et al. (25), neutrophils from whole blood preactivated with fMLP sequestered in coronary microvasculature in significantly larger numbers than the neutrophils from control blood. Furthermore, their work demonstrated that during early reperfusion, while ischemia-induced changes in the coronary microvasculature are sufficient to sequester neutrophils in the coronary capillaries, activation of neutrophils is necessary for their venular sequestration. This has significant implications in our study, because postcapillary venules are the primary sites for neutrophil extravasation into the tissues. It is therefore possible that neutrophils activated by allergen challenge sequestered in larger numbers in the venules, resulting in increased migration into the tissue and tissue injury.

In addition to neutrophils, differential activation of other inflammatory cell types in asthma, such as T and B lymphocytes, may also contribute to enhanced IRI. For example, using a lymphocyte-deficient mouse model, Yang et al. (35) have demonstrated that lymphocytes play a significant role in myocardial reperfusion injury. No significant lymphocyte infiltration was observed in our model, possibly due to the shorter reperfusion period. Resident mast cells and macrophages have also been shown to contribute to reperfusion injury in other tissues (8) and could have contributed to the increased tissue injury observed in the present study.

Alteration of the contractile properties in airway smooth muscle in asthma has been well established (29). Although there is no evidence confirming alteration of vascular smooth muscle contractile properties at present, the possibility exists that, in addition to the enhanced inflammatory cell infiltration, other vascular effects, such as enhanced vasospasm of the coronary microvasculature, might have contributed to the worse outcome of IRI in the asthma models.

These experiments used a 20-min occlusion period. Thirty minutes is a more common occlusion period in this model,
but most studies of IRI are focused on mechanisms to reduce injury, and the occlusion time is selected to produce a sufficiently large infarction to permit measurable decreases associated with treatment. In our experiments, we hypothesized that treatment would exacerbate injury, and the occlusion time was shortened to enable an accurate reflection of expanded injury. As expected, the size of the infarction in our model was less than what is typically reported for 30 min of ischemia. Lefer and colleagues have used both 20- and 30-min occlusion periods in murine experiments of I/R. They have reported average infarct sizes of 20.9 ± 3.4% with 20 min of occlusion (13, 14) and 42.5 ± 4.4% with 30 min of ischemia (14). Our results (27.7 ± 3.6%) compare favorably with these reports, especially when considering strain-specific differences that may be present in I/R responses in mice (7, 9).

Overall, observations in this murine model of asthma are very similar to our previous observations (7) in a rabbit model. The similarity leads to several conclusions. First, the effects of airborne allergen on susceptibility to ischemic injury are not species specific and, therefore, are likely to be applicable to other species, including humans. Second, dust mite was used for sensitization and challenge in the rabbit model, whereas ragweed was used in the murine model. Similar observations, despite two different antigens, suggest that the observed cardiovascular effects are due to underlying inflammation resulting from the allergen challenge rather than specific effects of the allergens. Third, in the rabbit model, I/R was done in animals ventilated with room air, and, hence, the possibility that hypoxia and resultant pulmonary vasoconstriction could have contributed to the worsening of myocardial tissue damage could not be excluded. However, the murine model was ventilated with 100% oxygen during I/R, and arterial blood gas measured during the procedure showed adequate partial pressure of oxygen, thereby ruling out hypoxia or pulmonary vasoconstriction. Similar outcomes, despite the absence of indicators of hypoxia, suggest that cardiovascular complications in the asthmatic models are due to underlying pathophysiological changes other than the complications of hypoxia, pulmonary vasoconstriction, and resultant pulmonary hypertension.

Despite the overall similarities, some differences were observed between the two studies. In the rabbit model, infarct size and neutrophil count were higher after systemic allergen boost. However, systemic-only sensitization in the mouse model (RW/NS) showed no effect on the parameters studied. This can be explained by the fact that the rabbit model was a chronic model, where systemic sensitization was initiated 24-h postnatal and continued at 1-mo intervals up to 6 mo. The extended sensitization may have augmented the systemic inflammation in this model. In contrast, the murine model is an acute model with only three systemic injections, and, hence, the systemic only inflammatory effects were probably not evident.

In conclusion, although cardiovascular complications are frequently reported in cases of asthma, most complications are attributed to the chronic drug therapy. With mounting evidence of the presence of a systemic inflammation in asthma, it is possible that inflammation in asthma alters parameters that lead to adverse effects on the cardiovascular system. Results from this study indicate that, in a murine model, inflammatory responses associated with asthma enhance expression of attachment molecules in the coronary vasculature and activate circulating neutrophils, resulting in recruitment of highly activated neutrophils to the infarct zone during an acute I/R event, thereby enhancing tissue destruction.
ACKNOWLEDGMENTS

We thank Howard Stallings, Jeremy Miles, Caitlin Van Scott, Catherine Lust, and Emily Cozzi for technical assistance.

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