Eosinophils From Hypereosinophilic Patients Damage Endocardium of Isolated Feline Heart Muscle Preparations

Ajay M. Shah, MB, MRCP, Dirk L. Brutsaert, MD, PhD, Ann L. Meulemans, DRs, Luc J. Andries, DRs, and Monique Capron, MD

Persistent eosinophilia in humans is often associated with endocardial damage to the heart, but a causal relation has not been established. We investigated the effect of eosinophils and eosinophil supernatants obtained from eight hypereosinophilic patients on the contractile performance and endocardial morphology of isolated, electrically stimulated cat papillary muscle preparations \(n=16\). All these eosinophil suspensions contained high proportions of "hypodense" or "activated" cells. Eosinophils \(5-15 \times 10^6/10 \text{ ml organ bath}\) or eosinophil culture supernatants (prepared by overnight incubation at \(37^\circ\text{C}\)) when added to papillary muscles produced acute changes in contractile behavior of these muscles identical to the previously reported effects of selective endocardial damage: a reduction in time to peak isometric twitch tension causing a reduction in peak isometric tension but with no significant reduction in rate of tension development or in maximum unloaded shortening velocity. All of these muscle preparations showed severely damaged endocardium at scanning electron microscopy. Addition of eosinophils from hypereosinophilic patients to muscles with selectively damaged endocardium (by previous transient [1-second] exposure to 1\% Triton X-100) produced no further change in contractile performance. No significant change in contractile performance or endocardial morphology of papillary muscles \(n=16\) was observed after addition of eosinophils \(7.5-10 \times 10^6\) or neutrophils \(8-15 \times 10^6\) from normal subjects or of cell-free culture medium. Thus, activated human eosinophils produce specific morphological and functional changes suggestive of specific damage to endocardium of isolated feline cardiac muscle. (Circulation 1990;81:1081-1088)

Persistent peripheral blood hypereosinophilia is associated with a high incidence of tissue damage.\(^1,2\) The cardiac disease associated with eosinophilia is typically characterized by damage to the endocardium and adjacent myocardium-endomyocardial fibrosis.\(^3,4\) It has been suggested that there is a causal link between the presence of eosinophilia and the occurrence of cardiac damage. As a result of release of granule products, eosinophils have a high cytotoxic potential against parasites but also a strong cytotoxic potential against mammalian target cells (including cultured human atrial myocytes).\(^5,6\) So-called "hypodense" and "degranulated" eosinophils, which are believed to be "activated," and eosinophil granule proteins have been found in high concentration in the peripheral blood of patients with clinical cardiac damage and in close association with these cardiac lesions.\(^2,7,8\) Tai et al\(^9\) have reported that eosinophil supernatants and purified eosinophil granule products impair the metabolic performance of isolated rat cardiomyocytes. Direct investigation of the acute effects of eosinophils and their secretion products on intact multicellular heart preparations has not, to our knowledge, been reported.

Brutsaert and coworkers\(^10-14\) have recently reported that the endocardium exerts a unique modulating influence on the contraction of isolated cardiac muscle. Selective damage to endocardium results in very characteristic changes in contractile twitch behavior: an earlier onset of isometric tension decline with reduction in peak isometric tension but a relatively minor reduction in the rate of tension development and no reduction in maximum unloaded shortening velocity.
TABLE 1. Characteristics of Hypereosinophilic Subjects

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Clinical features</th>
<th>Blood eosinophil count (% of eosinophils)</th>
<th>&quot;Hypodense&quot; eosinophils (%)</th>
<th>Purity of eosinophil preparation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M.R.</td>
<td>32</td>
<td>F</td>
<td>Hypereosinophilic syndrome</td>
<td>Neurological</td>
<td>3.23</td>
<td>81</td>
<td>70</td>
</tr>
<tr>
<td>2 B.D.</td>
<td>38</td>
<td>F</td>
<td>Taeniasis and trichinosis</td>
<td>Diarrhea, skin rash, respiratory</td>
<td>0.95</td>
<td>32</td>
<td>96</td>
</tr>
<tr>
<td>3 J.B.</td>
<td>61</td>
<td>M</td>
<td>“Myeloproliferative” hypereosinophilic syndrome</td>
<td>Skin rash, myalgia, anemia</td>
<td>8.44</td>
<td>99</td>
<td>88.5</td>
</tr>
<tr>
<td>4 F.D.</td>
<td>63</td>
<td>M</td>
<td>Postparasitic hypereosinophilia</td>
<td>Colitis, asthenia</td>
<td>1.52</td>
<td>59.6</td>
<td>66</td>
</tr>
<tr>
<td>5 M.G.</td>
<td>46</td>
<td>F</td>
<td>Churg-Strauss syndrome</td>
<td>Asthma, nasal polyps, neuropathy</td>
<td>3.07</td>
<td>48.1</td>
<td>96</td>
</tr>
<tr>
<td>6 M.T.</td>
<td>36</td>
<td>F</td>
<td>Atopic hypereosinophilic syndrome</td>
<td>Urticaria</td>
<td>1.56</td>
<td>36.4</td>
<td>94</td>
</tr>
<tr>
<td>7 R.J.</td>
<td>60</td>
<td>M</td>
<td>Filariasis</td>
<td>Pruritus</td>
<td>3.43</td>
<td>87.6</td>
<td>68</td>
</tr>
<tr>
<td>8 B.B.</td>
<td>44</td>
<td>M</td>
<td>“Myeloproliferative” hypereosinophilic syndrome</td>
<td>Endomyocardial fibrosis, diarrhea</td>
<td>7.20</td>
<td>84.7</td>
<td>94</td>
</tr>
</tbody>
</table>

We have studied the acute effects of activated eosinophils and eosinophil supernatants obtained from patients with hypereosinophilia on the contractile performance and endocardial morphological integrity of isolated cat cardiac papillary muscle preparations. As controls, the effects of eosinophils or neutrophils purified from normal subjects also were studied.

Methods

Subjects

Eosinophils and eosinophil supernatants were obtained from eight patients with hypereosinophilia. The clinical characteristics and hematologic data for these patients are shown in Table 1. None of the patients had received any treatment for at least 6 months before collection of blood for eosinophil purification. Only one subject (patient 8) had overt clinical evidence of cardiac disease. All patients had eosinophil counts of more than 0.9×10^9/l and evidence of eosinophil activation as assessed by the presence of a high proportion of "hypodense" eosinophils.6,15,16 Eosinophils and neutrophils were also obtained from five normal subjects.

Isolation of Eosinophils and Neutrophils

Peripheral blood eosinophils and neutrophils were isolated as described previously.15 Briefly, leukocytes were recovered from heparinized venous blood by dextran sedimentation of erythrocytes and were washed in minimum essential medium (MEM; Difco, Detroit, Michigan) containing 10% inactivated fetal calf serum (FCS). Cells suspended in MEM-FCS at 5×10^6 cells/ml were then layered on discontinuous metrizamide gradients (Nyegaard Co., Oslo, Norway), and cell fractions were collected from each gradient and interface. Percent purity and morphology of eosinophils or neutrophils was assessed using Giemsa-stained cytocentrifuge preparations and cell viability by trypan blue dye exclusion. All suspensions used for eosinophil experiments contained at least 65%, and usually more than 80%, eosinophils; neutrophil suspensions contained at least 90% neutrophils.

Eosinophil Supernatants

Purified eosinophils (5–10×10^6/ml) in MEM-FCS were incubated overnight at 37°C in a 5% CO₂ in air atmosphere. The supernatant was collected by centrifugation and stored at -20°C until use. Viability of eosinophils after preparation of the supernatants, evaluated by ethidium bromide, was very high. In addition, it has been previously shown that there is no alteration in protein expression when freshly purified eosinophils are compared with overnight cultured cells.17 Both purification of eosinophils or neutrophils and preparation of overnight culture supernatants were performed in the same place. Freshly purified eosinophils and culture supernatants or neutrophils were transported on ice to perform the experiments on physiology and electron microscopy (total transport time, approximately 1 hour).

Papillary Muscle Preparations

Right ventricular papillary muscles (n=35) from 15 pentobarbitone-anesthetized cats were mounted in 10-ml organ baths containing Krebs-Ringer solution of (mM) NaCl 118, NaHCO₃ 24, KCl 4.7, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.2, glucose 4.5, CaCl₂·2H₂O 1.25, pH 7.4) at 35°C and bubbled with a 95% O₂-5% CO₂ gas mixture. Muscles were attached to a length-tension transducer12 and stimulated electrically at 0.2 Hz and a voltage approximately 10% above threshold via two longitudinally arranged platinum electrodes. The basic characteristics of these muscles and the interventions studied are shown in Table 2.

The following contractile parameters were measured at l_max (the muscle length at which active tension development was maximal): total peak isometric twitch tension (TT), peak rate of isometric tension development (+dT/dt), time to peak isomet-
TABLE 2. Basic Characteristics of Cat Papillary Muscles

<table>
<thead>
<tr>
<th>Intervention studied</th>
<th>n</th>
<th>(L_{\text{max}}) (mm)</th>
<th>TT (mN/mm²)</th>
<th>+dT/dt (mN/mm²/sec)</th>
<th>tTT (msec)</th>
<th>RT(\frac{1}{2}) (msec)</th>
<th>(V_{\text{max}}) (L(\text{max}^\prime)/sec)</th>
<th>PS (proportion (L_{\text{max}}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Activated&quot; eosinophils and supernatant</td>
<td>19</td>
<td>8.1±0.79</td>
<td>50.5±12.19</td>
<td>276.2±74.04</td>
<td>268.8±18.02</td>
<td>421.1±27.61</td>
<td>1.32±0.120</td>
<td>0.14±0.009</td>
</tr>
<tr>
<td>Normal eosinophils</td>
<td>5</td>
<td>7.4±0.40</td>
<td>44.4±7.29</td>
<td>261.6±53.81</td>
<td>265.2±17.35</td>
<td>421.2±22.81</td>
<td>1.28±0.130</td>
<td>0.13±0.004</td>
</tr>
<tr>
<td>Normal neutrophils</td>
<td>5</td>
<td>6.8±0.38</td>
<td>44.5±6.27</td>
<td>258.2±51.43</td>
<td>271.8±19.69</td>
<td>420.6±23.78</td>
<td>1.37±0.215</td>
<td>0.14±0.009</td>
</tr>
<tr>
<td>MEM-FCS</td>
<td>6</td>
<td>7.3±0.78</td>
<td>36.0±6.00</td>
<td>198.5±50.15</td>
<td>278.7±29.96</td>
<td>401.3±33.66</td>
<td>1.17±0.176</td>
<td>0.11±0.012</td>
</tr>
</tbody>
</table>

\(L_{\text{max}}\), muscle length at which active tension development was maximal; TT, total peak isometric twitch tension; +dT/dt, peak rate of isometric tension development; tTT, time to peak isometric twitch tension; RT\(\frac{1}{2}\), time to half isometric relaxation; \(V_{\text{max}}\), maximum velocity of unloaded shortening; and PS, peak isotonic shortening.

Values are mean±SEM at 35° C and 1.25 mM Ca²⁺.

ric twitch tension (tTT), time to half isometric relaxation (RT\(\frac{1}{2}\)), peak isometric shortening (PS), and maximum velocity of unloaded shortening (\(V_{\text{max}}\)). Results were normalized for muscle cross-sectional area and \(L_{\text{max}}\). Only preparations with resting tension less than 15% of total developed tension were studied. Statistical analysis of changes in contractile performance was by the Student’s paired \(t\) test.

**Experimental Protocol**

After a 2–3-hour equilibration period, single doses of eosinophil or neutrophil suspension (5–15×10⁶ cells; volume, ≤500 µl), eosinophil supernatant (obtained from 5–15×10⁶ cells) or cell-free culture medium (with FCS) were added to organ baths containing a papillary muscle preparation, and any changes in contractile performance were measured after establishment of a stable response. Thirty minutes after addition of solution, muscles were fixed in their working position for scanning electron microscopy. Only one intervention was studied per muscle preparation.

**Electron Microscopy**

Preparations were fixed in 1% glutaraldehyde in 0.15 M cacodylate buffer for at least 2 hours and postfixed with 0.2% osmium tetroxide in cacodylate buffer for 1 hour. They were then dehydrated in an ascending series of acetone, critical point–dried in a Balzers apparatus, mounted on aluminum stubs, and coated with a 15-nm thick layer of gold. All specimens were examined in a Leitz AMR-1200-B microscope at 15 kV by one of us (L.A.) who was unaware of the experimental intervention tested. The morphology of the endocardium was assessed for integrity of endothelial cell membranes and nuclei, presence of endothelial intercellular spaces, and exposure of and damage to the basement membrane underlying the endocardial endothelium and then was quantified by assigning a score of 0–4+ for each of the above. A score of 0 meant no detectable abnormality, and a score of 4+ meant severe abnormality or damage.

**Results**

**Effects on Contractile Performance**

Addition of eosinophils or eosinophil supernatants from the hypereosinophilic patients to cat papillary muscles (\(n=16\)) resulted, usually within 15 minutes, in a characteristic alteration of contractile twitch (Figure 1). The tTT decreased by approximately 7% (\(p<0.005\)), resulting in an earlier onset of isometric twitch tension decline, and there was a 9% reduction in TT (\(p<0.01\)). RT\(\frac{1}{2}\) also decreased by 5% (\(p<0.01\)), thus contributing to the reduced duration of twitch. +dT/dt was minimally reduced, and there was no reduction in \(V_{\text{max}}\). This characteristic pattern of "negative inotropic" effect—identical to the effect of selective endocardial damage—was observed after addition of eosinophils or eosinophil supernatant and at all doses tested between 5 and 15×10⁶ cells. The mean changes in all measured contractile parameters are depicted in Figure 1.
TABLE 3. (Percent) Change in Contractile Parameters of Cat Papillary Muscles

<table>
<thead>
<tr>
<th>Intervention</th>
<th>TT (mN/mm²)</th>
<th>+dT/dt (mN/mm²/sec)</th>
<th>tTT (msec)</th>
<th>RT½ (msec)</th>
<th>Vₘₐₓ (Lₘₐₓ/sec [×10⁻²])</th>
<th>PS (Lₘₐₓ/sec [×10⁻²])</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Activated&quot; eosinophils and supernatant</td>
<td>-4.2±1.36*</td>
<td>-8.0±6.78</td>
<td>-17.5±4.3*</td>
<td>-21.5±4.4*</td>
<td>-0.5±1.5</td>
<td>-0.4±0.1</td>
</tr>
<tr>
<td>Normal eosinophils</td>
<td>0.8±0.51</td>
<td>4.5±4.31</td>
<td>-2.4±1.1</td>
<td>4.2±5.0</td>
<td>0.2±2.7</td>
<td>0.0±0.1</td>
</tr>
<tr>
<td>Normal neutrophils</td>
<td>0.7±0.43</td>
<td>1.8±2.35</td>
<td>-3.2±3.4</td>
<td>-4.8±3.9</td>
<td>3.8±3.6</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>MEM-FCS</td>
<td>0.1±0.14</td>
<td>4.5±4.31</td>
<td>0.2±1.9</td>
<td>-0.5±1.7</td>
<td>-1.0±1.0</td>
<td>0.1±0.1</td>
</tr>
</tbody>
</table>

Values are given as mean±SEM of raw data, and percent changes are between brackets.
Lₘₐₓ, muscle length at which active tension development was maximal; TT, total peak isometric twitch tension; +dT/dt, peak rate of isometric tension development; tTT, time to peak isometric twitch tension; RT½, time to half isometric relaxation; Vₘₐₓ, maximum velocity of unloaded shortening; and PS, peak isotonic shortening.

*p<0.01 cf. baseline values before intervention.

parameters (pooled data from both groups) are shown in Figure 1 and Table 3.

Addition of eosinophils (7.5–10×10⁶ cells) or neutrophils (8–15×10⁶ cells) obtained from normal subjects or of 500 µl MEM-FCS medium produced no significant change in papillary muscle contractile behavior (Table 3).

Eosinophils (7.5×10⁶ cells per experiment) from patients 7 and 8 were added to three cat papillary muscle preparations where endocardium had already been selectively damaged by transient (1-second) exposure to 1% Triton X-100 (Sigma, St. Louis, Missouri) as described previously. In these preparations, no significant further change in contractile perfor-

FIGURE 2. Scanning electron micrographs of cat papillary muscles. Intact endocardial endothelium is present in a control muscle after addition of culture medium (A) and in muscles after treatment with normal neutrophils (B) or normal eosinophils (C and D). The endothelial cells have intact cell membranes, intercellular borders (arrowheads), and small microvilli (A–D). Scale bars, 5 µm.
mance was observed during 30 minutes’ exposure to eosinophils (TT increased by 1.3±2.43%, TT by 0.1±0.17%, and RT½ by 0.9±1.90%, and Vmax decreased by 0.2±0.20% [mean±SD]).

**Scanning Electron Microscopy**

Normal endocardial endothelium of cat papillary muscle preparations mounted for up to 8 hours consists of a smooth continuous cell sheet of closely apposed cells with distinct intercellular borders, intact cell membranes, and a variable number of small microvilli. Specimens treated with eosinophil-free culture media (n=6; Figure 2A), normal eosinophils (n=5; Figures 2C and 2D), or normal neutrophils (n=5; Figure 2B) had similar appearances—predominantly normal endocardial endothelium but also the very occasional cell with a damaged cell surface (Table 4).

Muscle preparations (n=16) exposed to eosinophils or eosinophil supernatants from hypereosinophilic patients had severely damaged endocardium (Figure 3 and Table 4). Both eosinophils (Figures 3A and 3B) and their supernatants (Figures 3C and 3D) typically caused a heavily extracted appearance of the endocardial cells. This extraction of the endocardial cells ranged from multiple holes in the cell membrane to a “pepper-pot” appearance and a more severe, nearly complete extraction that left only some cytoplasmic fragments and nuclei. Despite the severe damage to the endocardial cells, their cell shape was usually unaffected, and intercellular borders were still present (Figures 3A–3C). Zones where endocardial cells were less extracted showed many abnormal intercellular spaces and areas where the underlying basement membrane was exposed, presumably due to damage and detachment of endocardial cells. In these areas, the basement membrane also showed evidence of damage. The pattern of endocardial damage observed was similar for preparations treated with eosinophils or with eosinophil supernatants (Table 4).

**Discussion**

In this study, eosinophils and eosinophil culture supernatants from hypereosinophilic patients pro-
duced characteristic acute changes in contractile behavior in association with severe morphological damage to endocardium of cat papillary muscle preparations. Eosinophils or neutrophils from normal volunteers and cell-free culture medium produced no significant functional or morphological changes. We have also previously found that aggregating blood platelets do not damage endocardium under these conditions. 14

Damage to endocardium by eosinophils was probably mediated by eosinophil secretion products because eosinophil supernatants had the same effects as intact cells. The crude eosinophil supernatants were negative for proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and it would be necessary to concentrate them before attempting identification of the products responsible for the endocardial damage by a more sensitive assay. One might speculate that eosinophil cationic protein, eosinophil peroxidase, or eosinophil-derived neurotoxin might be responsible for the damage (rather than the eosinophil major basic protein, which is less soluble) as is suggested by previous studies on single cells. 6,18 It has also been shown that eosinophil cationic protein may mediate membrane damage by forming transmembrane pores. 19 The characteristic morphology of the damaged endocardial cell membranes is consistent with this possibility.

The possible mechanisms of release of these substances and of induction of cell damage were not explored in this study. Because no stimulating agent was used, it is likely that there was spontaneous release of eosinophil products perhaps due to a high state of activation of eosinophils: all eosinophil suspensions obtained from hyper eosinophilic patients contained high proportions of the hypodense eosinophils, which are believed to be activated. 6,15,16 The possibility of cell damage mediated by antibodies cytophilically bound to the eosinophil cell surface (as shown for IgE 20) should also be considered. However,
the damaging effects of eosinophil supernatants would then have to be explained on the basis of spontaneous antibody release into the supernatant. IgE molecules can be experimentally eluted from the eosinophil FcE receptor by acid pH treatment but not spontaneously.21

The changes in contractile behavior produced by activated eosinophils and their supernatants are identical to those resulting from selective damage to endocardium of isolated cat papillary muscle by gentle mechanical abrasion or by transient exposure to the detergent Triton X-100,12 suggesting that these eosinophil soluble mediators similarly induce selective damage to the endocardium without damaging myocardium. Our data showing no significant reduction in +dT/dt or in V \(_{\text{max}}\) also provide functional evidence of myocardial integrity. V \(_{\text{max}}\) is a measure of the force-velocity relation22 of the muscle and is believed to represent the life cycle of a single crossbridge between actin and myosin: it would be expected to decrease if myocardium were damaged. Similarly, the rate of tension development usually decreases significantly when myocardium is damaged. Furthermore, addition of eosinophils to preparations where endocardium had already been selectively damaged produced no further acute change in contractile performance. However, because the myocardium was not systematically examined by transmission electron microscopy of all specimens, no further conclusions can be made regarding morphological integrity of the myocardium.

It is possible that endocardium is selectively damaged by activated eosinophils because it is more susceptible to damage than the myocardium. Alternatively, the relatively short time of exposure (30 minutes) in this study might have restricted damage to superficial areas of the papillary muscles. The clinical pattern of eosinophil-associated cardiac disease in humans is also one of predominantly endocardial damage supporting the hypothesis that endocardium is more prone to damage than myocardium. An interesting and relevant question is whether acute endocardial damage in vivo produces acute changes in contractile behavior of subjacent myocardium similar to the changes observed in this study and whether these might cause clinically significant hemodynamic disturbance. Although the magnitude of such an effect would be small, significant hemodynamic changes could occur as a result of increased nonuniformity of contractile behavior. However, the phenomenon of endocardial modulation of myocardial contractile behavior in vivo is still under investigation. Furthermore, the mechanisms responsible for the effect of endocardium in isolated cardiac muscle are also speculative at present.24,25

The results of this study show that the endocardium of isolated cardiac papillary muscle is specifically damaged by mediators from activated eosinophils and results in a characteristic change in the pattern of contraction and relaxation. The cat papillary muscle preparation may be a useful simple and sensitive model to further investigate the mechanical effects and mechanisms of eosinophil-induced cardiac damage.

Acknowledgments

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**KEY WORDS**  - eosinophils  - endocardial endothelium  - endomyocardial fibrosis  - hypereosinophilia
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