Synthesis of Interleukin-5 by Activated Eosinophils in Patients With Eosinophilic Heart Diseases

By Pierre Desreumaux, Anne Janin, Sylvain Dubucquoi, Marie-Christine Copin, Gérard Torpier, André Capron, Monique Capron, and Lionel Prin

Eosinophilic endomyocardial disease represents a major evolutive risk in chronic eosinophilia-associated disorders. Eosinophil granule proteins appear to be involved in cardiac injury, but the mechanisms leading to eosinophil infiltration and degranulation are not clear. Interleukin-5 (IL-5) has been recently shown to be produced by eosinophils and might play a role in both chemoattraction and degranulation of eosinophils. In four cases of eosinophilic diseases with severe cardiac failure, we evaluated the proportion of eosinophil phenotypes and the serum levels of eosinophil cationic protein (ECP) and soluble IL-2 receptor (sIL-2R), markers of disease activity in the hypereosinophilic syndromes. All four patients showed a markedly increased proportion of hypodense eosinophils with elevated serum ECP and sIL-2R levels. In all four patients, extracellular deposition of eosinophil granule proteins and features of eosinophil activation were observed in cardiac tissues. The synthesis of IL-5 by eosinophils was detected in myocardial sections and blood cells by in situ hybridization and by immunostaining with a monoclonal antibody against human IL-5. Sixty percent to 90% of tissue eosinophils expressed IL-5 mRNA and IL-5 protein. These data suggest that IL-5 can be produced by eosinophils at the sites of myocardial tissue damage and might participate in local eosinophil activation.

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MATERIALS AND METHODS

Patients. The study included four patients with eosinophilic diseases associated with severe cardiac failure. For each patient, blood and tissue markers of disease activity were analyzed after informed consent was obtained. A 47-year-old woman (case no. 1) and 50-year-old man (case no. 2), both fulfilling the diagnostic criteria for idiopathic hypereosinophilic syndrome (HES), developed chronic endomyocardial fibrosis. Conventional treatment with prednisone (30 to 60 mg/d) and hydroxyurea (1 to 2 g/d) was started. Four months after the initiation of therapy, aggravation of heart failure was appreciated by clinical and biologic parameters, including cardiac catheterization, echocardiography, and electrocardiography. α-IFN therapy was then started after informed consent was obtained. A daily dose of 5 million units for 5 days per week, administered subcutaneously, was administered during the time of the study. A 61-year-old woman (case no. 3) presented with dyspnea 4 days before admission. She fulfilled the diagnosis criteria of HES and developed chronic endomyocardial fibrosis. Despite treatment with prednisone (60 mg/d), cardiac decompensation was rapidly progressive and she died from intractable cardiac arrhythmias. A 7-year-old girl (case no. 4) presenting with dyspnea (60/min) and tachycardia (150/min) developed sudden cardiac deterioration related to a postmortem diagnosis of eosinophilic myocarditis. She never related having drug hypersensitivity. While emergency medica-

From the Centre d’Immunologie et Biologie Parasitaire, Institut Pasteur; and the Anatomie et Cytologie Pathologiques, Hôpital Calmette, CHU Lille, Lille, France.

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Address reprint request to Monique Capron, PhD, CIBP, Institut Pasteur, 1 rue du Pr A. Calmette, B.P. 245, 59019 Lille Cédex, France.

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Fig 1. Serial measurements of blood eosinophilia, ECP levels, and sIL-2R values in patients no. 1 and 2 before and after treatment with α-IFN. There was a significant association between sIL-2R values, ECP levels, and blood eosinophilia in patient no. 2.

sures were being instituted and prednisone treatment started, the child died with a pattern of infarction on electrocardiography.

Eosinophil purification procedure. The processing of blood samples for eosinophil purification was the same for the four patients. Blood eosinophils were purified by centrifugation on discontinuous metrizamide gradients, as previously described. Using this cell purification procedure, hypodense eosinophils that sediment in 20%, 22%, and 23% metrizamide solutions were separated from eosinophils that sediment in fractions of intermediate density (24% metrizamide solution) or high density zone (25% metrizamide solution). The degree of eosinophil purity and the yield of eosinophils recovered were evaluated for each fraction (cytocentrifuge smears and giemsa staining) and the percentage of hypodense eosinophils was calculated.

Measurement of ECP and soluble IL-2 receptor (sIL-2R). The serum levels of ECP were evaluated in the four patients. Ten milliliters of peripheral blood was collected by venipuncture for each patient at the time of biopsy or death and kept at 4°C before centrifugation at 850g for 10 minutes. Collected sera were stored at -20°C before analysis. ECP levels were determined by means of a commercially available double-antibody radioimmunoassay (ECP/RIA; Pharmacia, Uppsala, Sweden). Serum levels of sIL-2R were evaluated in the four patients using a sandwich enzyme immunoassay (Cell Free; T Cell Sciences, Inc, Cambridge, MA) as previously described.

Morphologic studies. Samples of endocardium and myocardium were obtained by cardiac biopsy for patients no. 1 and 2 and at necropsy in the two other patients (Table 1). Specimens were immediately fixed in fresh 4% paraformaldehyde/phosphate-buffered saline, embedded in paraffin, and further processed for histologic examinations. Alternatively, specimens were fixed in 1% glutaraldehyde/cacodylate, embedded in Epon, and processed for ultrastructural studies.

Indirect immunoenzymatic methods were used to detect the presence of granule cationic proteins and the Charcot Leyden crystal (CLC) with the following antibodies (Ab): EG2, a mouse monoclonal Ab (MoAb) directed against the secreted form of ECP, kindly given by Dr Po Chun Tai (St George's Hospital Medical School, London, UK); BMK-13, a mouse MoAb that binds to MBP (TEBU, Le Perray en Yvelines, France); a polyclonal rabbit anti-eosinophil-derived neurotoxin (EDN) kindly given by Dr P. Deviller (Hôpital Cardiovasculaire et Pneumologique Louis Pradel, Lyon, France); a polyclonal rabbit anti-CLC antiserum kindly provided by Dr G.J. Gleich (Mayo Clinic, Rochester, MN); and an antihuman IL-5 MoAb (Glaxo Research Group, Greenford, Middlesex, UK). The binding of these antibodies, used at 20 μg/mL for mouse MoAb and 1/50 dilution of rabbit antiserum, was detected by means of streptavidin and biotinylated horseradish peroxidase complex (Strept ABC complex/HRP; Dakopatts, Glostrup, Denmark) or with complexes of calf intestinal alkaline phosphatase and
mouse monoclonal anti-alkaline phosphatase (APAAP; Dakopatts; 1:50 concentration).

*In situ hybridization.* The cDNA for human IL-5 was subcloned into the Blue Script vector by standard techniques. Radiolabeled RNA probes were prepared as previously described. In brief, linearized plasmid was used as the template for the synthesis in vitro of a 35S-labeled RNA probe (Amersham-France, Les Ullis, France) complementary to the cellular IL-5 mRNA (antisense probe). RNA was also transcribed in the opposite direction and used as a negative control (sense probe). Antisense or sense probes (4,150 cpm/mm²) were hybridized with cardiac samples as previously described. To inhibit nonspecific binding of 35S, tissues and cells were acetylated in 0.1 mol/L triethanolamine for 5 minutes and then in acetic anhydride 0.25% triethanolamine for 10 minutes before hybridization. To further avoid nonspecific binding to eosinophils, prehybridization was performed with a solution containing a nonradiolabeled S-UTP irrelevant probe for at least 2 hours at 42°C. Dithiothreitol was added to the hybridization buffer, and RNAse A was used for posthybridization washings. After development of the emulsion, tissue sections or cytospin preparations were then stained with May-Grunwald Giemsa for examination by light microscopy.

Blood hypodense eosinophils were collected by metrizamide centrifugation and the degree of eosinophil purity evaluated by May-Grunwald Giemsa differential counting on cytocentrifuged preparations. In all cases, eosinophil purity was greater than 95%. These cells were resuspended at a concentration of 0.8×10⁶ cells/mL of Hanks’ Balanced Saline Solution and loaded onto gelatin-coated slides (0.8×10⁶ cells/slides) by cytocentrifugation. The cytospin preparations were immediately fixed for 20 minutes in 4% paraformaldehyde and stored at –20°C until use. In situ hybridization was then performed using the same technical conditions and the same probes as for tissue sections.

*Statistical analysis.* The relationships between ECP levels, sIL-2R levels, and blood eosinophilia were determined using the non-parametric Spearman’s rank correlation test (P < .05 was considered significant).

**RESULTS**

**Blood markers of disease activity.** To investigate the state of activation of blood eosinophils, various biologic parameters were evaluated. First, the separation of eosinophil phenotypes was performed by centrifugation of blood leukocytes on discontinuous metrizamide gradients, as previously described. Using the same separation procedure for the four patients, hypodense eosinophils, sedimenting in fractions of low density (20% to 23% metrizamide, density [d] < 1.135) could be collected and their numbers compared with those of eosinophils with intermediate or high density (>23% metrizamide, d > 1.135). As illustrated in Table 2, a very high proportion of hypodense eosinophils (92% to 97%) was observed in all four patients compared with that in historical controls.

The presence of one eosinophil granule cationic protein, ECP, in the serum or biologic fluids of patients after eosinophil degranulation can be a valuable and objective parameter in monitoring disease activity, particularly after treatment. Results shown in Table 2 indicated significantly increased serum levels of ECP in the four patients compared with normal values. Because several studies have suggested a relationship between T-lymphocyte stimulation and eosinophil activation, and because it has been recently shown that patients with HES have increased levels of sIL-2R," serum levels of IL-2R were evaluated. As shown in Table 2, very high values of sIL-2R were detected in the serum of the four patients.

The deterioration of heart failure in two patients (cases no. 1 and 2) despite conventional treatment (prednisone/hydroxyurea) led to the initiation of α-IFN therapy. Such therapy resulted in a dramatic beneficial response, with a marked improvement in cardiac function. As shown in Fig 1, serial measurements of various parameters (blood eosino-
philia, ECP, and sIL-2R) showed a parallel decrease of blood eosinophilia, ECP levels, and sIL-2R levels during α-IFN therapy. In the case of patient no. 2, a positive correlation could be established during α-IFN therapy between serum ECP levels and eosinophilia (r = .764; P < .01), between ECP levels and sIL-2R values (r = .537; P < .005), and between serum sIL-2R values and eosinophilia (r = .698; P < .01), indicating a significant association between these three parameters.

Tissue markers of disease activity. Morphologic studies (Table 1) showed that, in the case of endomyocardial biopsies (cases no. 1 and 2), eosinophils were found in edematous interfibrillar areas. There were no other inflammatory cells, no fiber vacuolisation, and no vasculitis. Electron microscopy showed eosinophils surrounded by numerous free granules due to lysis of the cytoplasm (Fig 2). Some granules showed an inverted density, indicating a state of eosinophil activation. Myocardial fibres were not damaged.

In cases no. 3 and 4, necropsy examination of the heart showed a voluminous adhesive thrombus within the left ventricle (Fig 3). No valvular damage was observed. No evidence of thrombosis was found upon dissection of the coronary vessels. Histologically, eosinophils were numerous, particularly in the endomyocardial area where the thrombus was organized. Fibrin deposits were found within the lumen of some intramyocardial vessels. Large areas of myocardial necrosis were found in case no. 3; perivascular fibrosis was found in case no. 4. Electron microscopy showed alterations of eosinophil granules and cytoplasm, whereas myocardial fibers were damaged with loss of myofilaments or complete necrosis. CLC were numerous within the thrombus and adjacent endocardium.

In cases no. 1 and 2, immunostaining showed extracellular deposits of the four eosinophil proteins, with a predominance of MBP and CLC in edematous interfibrillar areas. In cases no. 3 and 4, immunostaining showed extracellular deposition of all four eosinophil proteins (ECP, MBP, EDN, and CLC). Very few intact eosinophils were found on histologic sections (Fig 4A), whereas ECP deposition was particularly intense in the endomyocardial area of thrombus adhesion (Fig 4B).

Synthesis of IL-5 by blood and cardiac eosinophils. Positive in situ hybridization signals were observed on numerous cells infiltrating myocardial sections of the four patients only with the antisense IL-5 RNA probe and not with the sense probe. Similar label intensity was obtained in the myocardium of the four patients. Examination of the tissues at a higher magnification showed that same-labeled cells had a polylobulated nucleus (Fig 5A). To detect the presence of IL-5 protein in eosinophils from these patients, immunostaining of cardiac tissues was performed using anti-human IL-5 MoAb (Glaxo Research Group) and the APAAP technique described above. Results of one representative experiment performed on patient no. 4 show an intense staining of cells with a polylobulated nucleus with anti-human IL-5 (Fig 5B). An overall study performed on the myocardial sections of all four patients showed that approximately 60% to 90% cardiac eosinophils were positively labeled both by in situ hybridization and by immunostaining. Not only tissue cells but also highly purified blood eosinophils from the same patients were submitted to in situ hybridization with the IL-5 probe and human-IL-5 MoAb. As shown in Fig 5C, eosinophils expressed IL-5 mRNA, whereas no positive signal was detected with the control IL-5 sense probe (Fig 5D), confirming the idea that tissue and blood eosinophils could express the IL-5 mRNA. Anti-human–IL-5 MoAb used on the same cytopreparations of purified blood eosinophils showed an intense staining in the cytoplasm of eosinophils (Fig 5E), whereas no positive signal was detected with an irrelevant antibody on the same cytopreparations of purified blood eosinophils (Fig 5F). This finding confirmed the idea that tissue and blood eosinophils could produce the IL-5 protein.

Because blood eosinophil counts returned to normal levels after treatment with α-IFN and cardiac biopsy could not be repeated in treated patients, the effect of α-IFN on IL-5 synthesis by blood and tissue eosinophils could not be appreciated in these patients after treatment.
that eosinophils could present signs of activation in eosinophilic heart disease. Previous findings have suggested the presence of elevated numbers of blood eosinophils together with eosinophil infiltration in the cardiac tissue. Several studies have shown that eosinophils could present signs of activation in eosinophilic disorders associated with visceral involvement. Increased blood numbers of hypodense, degranulated, and vacuolated eosinophils have been indeed found in these patients. The cytotoxic potential of hypodense eosinophils and their basic granule proteins has been previously reported. Cardiac damage might be related to the toxic effects of activated eosinophils, because immunohistochemical studies have shown the deposition of eosinophil cationic granule proteins in endomyocardial lesions of patients with HES, in acute necrotizing eosinophilic myocarditis, and in syndromes associated with fibrosis. In the present work, markers of eosinophil activation were detected both in the blood and in the cardiac tissue of four patients with eosinophilic heart disease. However, the mechanisms responsible for activation and release of toxic mediators remain to be identified. The participation of activated blood T lymphocytes has been postulated, because very high levels of sIL-2R can be detected in the sera of patients with HES associated with visceral involvement. It has been recently shown that the serum sIL-2R level is a sensitive and reliable quantitative marker of mononuclear cell activation. In the present study, changes in serum sIL-2R values correlated with the absolute number of blood eosinophils and serum ECP levels throughout evolution of the eosinophilic disease. sIL-2R might arise from eosinophils themselves, as we have previously reported that hypodense eosinophils can express the IL-2R α-chain on the cell surface. Moreover, it has been recently shown that IL-5 can also be produced by cell types other than T cells, including mast cells, Reed-Sternberg cells, and eosinophils. Because no inflammatory cell type other than eosinophils was found in cardiac tissue, it was interesting to examine whether activated eosinophils from patients with eosinophilic cardiac disease might secrete IL-5 in humans, IL-5 appears to exert its effects primarily on eosinophils by enhancing their terminal differentiation and enhancing that process of degranulation. Thus, locally synthesized IL-5 might activate eosinophils and stimulate the release of toxic mediators through an autocrine mechanism.

Table 1. Results of Histological Studies Performed on Cardiac Biopsies (B) and Necropsies (N)

<table>
<thead>
<tr>
<th>Type of Specimens</th>
<th>Months between Onset of Disease and B or N</th>
<th>Heart Fiber Necrosis</th>
<th>Thrombus</th>
<th>Fibrosis</th>
<th>Site of Eosinophil Infiltrate</th>
<th>Ultrastructural Aspects of Eosinophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1 B</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Interfibrillar</td>
<td>Cytoplasmic lysis without nuclear picnosis</td>
</tr>
<tr>
<td>Case 2 B</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Interfibrillar</td>
<td>Cytoplasmic lysis without nuclear picnosis</td>
</tr>
<tr>
<td>Case 3 N</td>
<td>14</td>
<td>0</td>
<td>++</td>
<td>++</td>
<td>Perivenular</td>
<td>ND</td>
</tr>
<tr>
<td>Case 4 N</td>
<td>1</td>
<td>++</td>
<td>++</td>
<td>0</td>
<td>In thrombus and perivenular picnosis</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Biologic Parameters of Eosinophil Activation Obtained at the Time of Biopsy or Death

<table>
<thead>
<tr>
<th>Eosinophils (×10^9/L)</th>
<th>Hypodense Eosinophils (%)</th>
<th>ECP (µg/L)</th>
<th>sIL-2R (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal values</td>
<td>&lt;0.8</td>
<td>&lt;25</td>
<td>&lt;16</td>
</tr>
<tr>
<td>Case 1</td>
<td>3.6</td>
<td>95</td>
<td>445</td>
</tr>
<tr>
<td>Case 2</td>
<td>4.5</td>
<td>92</td>
<td>70</td>
</tr>
<tr>
<td>Case 3</td>
<td>4.9</td>
<td>97</td>
<td>170</td>
</tr>
<tr>
<td>Case 4</td>
<td>7.2</td>
<td>95</td>
<td>290</td>
</tr>
</tbody>
</table>

* Percent of total eosinophils.

DISCUSSION

The high morbidity and mortality of eosinophilic diseases are often related to heart failure. Chronic, subacute, or acute forms of cardiomyopathy have been described. One of their common features is the presence of elevated numbers of blood eosinophils together with eosinophil infiltration in the cardiac tissue. Previous findings have suggested that eosinophils could present signs of activation in eosinophilic disorders associated with visceral involvement. Increased blood numbers of hypodense, degranulated, and vacuolated eosinophils have been indeed found in these patients. The cytotoxic potential of hypodense eosinophils and their basic granule proteins has been previously reported. Cardiac damage might be related to the toxic effects of activated eosinophils, because immunohistochemical studies have shown the deposition of eosinophil cationic granule proteins in endomyocardial lesions of patients with HES, in acute necrotizing eosinophilic myocarditis, and in syndromes associated with fibrosis. In the present work, markers of eosinophil activation were detected both in the blood and in the cardiac tissue of four patients with eosinophilic heart disease. However, the mechanisms responsible for activation and release of toxic mediators remain to be identified. The participation of activated blood T lymphocytes has been postulated, because very high levels of sIL-2R can be detected in the sera of patients with HES associated with visceral involvement. It has been recently shown that the serum sIL-2R level is a sensitive and reliable quantitative marker of mononuclear cell activation. In the present study, changes in serum sIL-2R values correlated with the absolute number of blood eosinophils and serum ECP levels throughout evolution of the eosinophilic disease. sIL-2R might arise from eosinophils themselves, as we have previously reported that hypodense eosinophils can express the IL-2R α-chain on the cell surface. Moreover, it has been recently shown that IL-5 can also be produced by cell types other than T cells, including mast cells, Reed-Sternberg cells, and eosinophils. Because no inflammatory cell type other than eosinophils was found in cardiac tissue, it was interesting to examine whether activated eosinophils from patients with eosinophilic cardiac disease might secrete IL-5 in humans, IL-5 appears to exert its effects primarily on eosinophils by enhancing their terminal differentiation and enhancing that process of degranulation. Thus, locally synthesized IL-5 might activate eosinophils and stimulate the release of toxic mediators through an autocrine mechanism.

The majority of eosinophils from endomyocardial tissues of patients with eosinophilic heart disease expressed IL-5 mRNA and synthesized IL-5 protein. In all cases, eosinophils were activated as shown in cardiac biopsies by ultrastructural changes and by immunohistochemical staining with EG2, a MoAb that binds to the activated form of ECP. Therefore, the local synthesis of IL-5 in endomyocardial tissues might contribute to the proliferation and activation of eosinophils by enhancing the release of cationic proteins that could be involved in the progression of cardiac lesions. It has to be mentioned that, in a separate study, the levels of IL-5 secreted by purified blood eosinophils upon activation with various stimuli have been measured by an enzyme-linked immunosorbent assay and reached values up to 250 pg/1 × 10^6 cells, which are capable of inducing the proliferation of an IL-5-dependent B-cell line (Dubreucquoi et al submitted for publication). An autocrine mechanism in which stimulated eosinophils both release and respond to IL-5 could therefore be involved, because IL-5 membrane receptors have been described on hypodense blood and tissue eosinophils. Although they indicate that eosinophils might contribute to the local production of IL-5, these results do not imply that eosinophils represent the only source of IL-5, because previous studies have shown the production of IL-5 by T cells from patients with various HES. However, the present study did not allow us to assess the relative significance of IL-5 derived from T cells and eosinophils in the etiology of the disease because 95% of the cells infiltrating the lesions were eosinophils. The low percentage of infiltrating lymphocytes was not sufficient to perform statistically significant analysis. In a more recent study, we could show that both EG2-positive eosinophils and EG2-negative T cells expressed IL-5 mRNA and immunoreactive protein in the skin of patients with atopic dermatitis (Tanaka et al, submitted for publication).

In vitro functional studies of the effects of α-IFN on eosinophils have led to variable results. α-IFN suppresses eo-
sinophilic colony growth and decreases eosinophil peroxidase and hydrogen peroxide release in response to stimuli. However, it has been shown that α-IFN activates some eosinophil functions. After the establishment of its suppressive effects on granulocyte-macrophage colonies in vitro, α-IFN has been recently administered to patients with HES, resulting in dramatic clinical and hematologic improvement. More recently, a beneficial clinical response associated with a rapid decrease in sIL-2R levels and blood eosinophilia has been reported in six cases of malignant HES treated with α-IFN. In the present study, two patients were clinically improved after α-IFN therapy, with a significant decrease in blood eosinophil counts, in serum ECP, and in sIL-2R levels, all considered as useful indicators of disease activity in HES patients.

Experiments are now in progress to explore the effects of α-IFN on IL-5 synthesis by eosinophils from patients with eosinophilic heart diseases. The mechanisms determining local eosinophil accumulation and activation in tissues and particularly in cardiac tissues, in which eosinophils induce dramatic pathologic changes, have still to be explored. However, the present results suggest that IL-5 synthesized by eosinophils might play a major role in the chemotraction of eosinophils and the release of toxic mediators from eosinophils, providing, therefore, an important autocrine activation pathway.

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Synthesis of interleukin-5 by activated eosinophils in patients with eosinophilic heart diseases

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