A Case of Hypereosinophilic Syndrome Is Associated With the Expansion of a CD3−CD4+ T-Cell Population Able to Secrete Large Amounts of Interleukin-5

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Interleukin-5 (IL-5) is the major soluble factor able to mediate hypereosinophilia. We report a case of hypereosinophilic syndrome in which the presence of a population of CD3−CD4+ cells able to overproduce IL-5 was shown. The lack of CD3 and TCRAB expression on otherwise phenotypically normal mature T lymphocytes together with the absence of detectable TCRBV mRNA and clonal rearrangement of TCRB gene suggested that the abnormal lymphocyte population was the expression of a peripheral T-cell lymphoma with an indolent clinical course. Peripheral blood lymphocytes enriched in this population were able to secrete high levels of IL-5 but not of IL-4, and no IL-2 or interferon-γ, when stimulated in vitro with phytohemagglutinin and phorbol myristate acetate. The serum contained eosinophil survival factors whose activity was partially neutralized by a specific antihuman IL-5 antibody. This observation further emphasized the relationship between hypereosinophilic syndrome, IL-5, and T-cell lymphoproliferative disorders.

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The relationship between hypereosinophilia and T lymphocytes is well established and is mediated by the production of eosinophilopoietic cytokines. CD4+ lymphocytes are the most important source of granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and IL-5, three currently recognized cytokines active in promoting the development and differentiation of eosinophils in bone marrow. In mice, T-cell clones can be classified according to the pattern of cytokine secretion and there is good evidence that also human cells could be subdivided into these two profiles. Type 1 helper T-cell (Th1) clones secrete IL-2 and interferon-γ (IFN-γ), whereas type 2 (Th2) clones secrete IL-4 and IL-5; both secrete IL-3 and GM-CSF. Interestingly, these latter two cytokines also stimulate the development of other marrow-derived cells, whereas IL-5 in humans acts more specifically on eosinophils and is the major, and possibly the only, cytokine involved in the production of specific eosinophilia. Over the last few years it became evident that the IL-5 production plays a pivotal role in the development of hypereosinophilia both in the secondary forms associated with allergic diseases or parasitic infections and in the idiopathic syndrome. Interestingly, at least in vitro, these cytokines dramatically increase the life span of eosinophils by inhibiting their apoptotic cell death.

The association between hypereosinophilia and T-cell lymphoma is also well established and in some studies the production of IL-5 by neoplastic T cells has been shown. Recently, a case of clonal proliferation of Th2 cells in a man with the hypereosinophilic syndrome (HES) was reported. The investigators showed the expansion of a T-cell clone with the unusual phenotype CD3−CD4+ able to produce high levels of IL-4 and IL-5 and low levels of IL-2 and IFN-γ.

We report a similar case of HES in which the presence of a population of CD3−CD4+ cells able to overproduce IL-5 (but not IL-4) was shown.

Materials and Methods

Case report. A 70-year-old man with hypereosinophilia for the last 5 years has been investigated. His absolute eosinophil count ranged from 2,700 to 16,600/μL. He complained of generalized pruritus with periodic eruptions of papular elements that on histologic examination showed nonspecific follicular inflammation. A physical examination showed mild axillary and inguinal lymph node enlargement and a histologic examination showed a reactive lymphoid hyperplasia with marked infiltration of eosinophils and polyclonal plasmacells with no evidence of lymphoma. Polyclonal hypergammaglobulinemia (γ globulins: 41% of 9.6 g/dL of total serum proteins) was present with high IgG and IgM and normal IgA and IgE levels. Other laboratory parameters were normal as well as peripheral blood eosinophil morphology.

Bone marrow biopsy results showed marked eosinophila and moderate polyclonal plasmacytosis. Cytogenetic analysis showed a normal karyotype. Causes of secondary eosinophilia were ruled out, including allergic, parasitic, neoplastic, or autoimmune disorders, and a diagnosis of HES was made. Complications of longstanding hypereosinophilia, particularly cardiac, were not found, despite periodic clinical and echographic monitoring.

Surprisingly, despite a normal blood lymphocyte count, immunophenotypic studies showed that 38% of lymphocytes were CD3−CD4+.

The patient was treated with 6 methylprednisolone (6MP; 16 mg/d). After 1 month, cutaneous symptoms disappeared and the eosinophil level decreased to 177/μL. He is currently on chronic low-dose 6MP (20 mg/wk). The eosinophil level is in the range of 2,000 to 3,000/μL. The CD3−CD4+ population decreased from 38% to 33% after 1 month and is now 13% to 17%.

Cell preparation. Mononuclear cells from the patient and from an age-matched healthy volunteer with no history of allergy and normal lymphocyte populations were separated from the peripheral blood by Ficoll-Hyphaque density gradient centrifugation and washed three times in RPMI-1640 (GIBCO Laboratories, Grand Island, NY).

Depletion of CD3+ cells from peripheral blood mononuclear cells (PBMC) was obtained using negative selection with mouse IgG-
conjugated magnetic beads (Dynal, Oslo, Norway) precoated for 30 minutes at 4°C with CD3 monoclonal antibody (MoAb; OKT3; Ortho Diagnostic, Rantian, NJ), as recommended by the manufacturer; less than 4% of negatively selected PBMC expressed CD3 after this procedure, as assessed by immunofluorescence analysis. This population was enriched in CD3+ CD4+ cells (57%) and was therefore used in experiments of cytokine production as representative of the unusual population. For TCRBV mRNA analysis, CD4+ CD8- cells were further purified from CD3-depleted PBMC by positive selection with CD4-coated beads (Dynal); more than 92% of positively selected cells were CD4+ CD3-. For these experiments, CD3- cells, purified by positive selection (96% CD3+, 61% CD4+, and 36% CD8-), were used as representative of a normal T-cell population.

**Cell cultures.** All experiments were performed in complete medium supplemented with 10% heat-inactivated fetal calf serum (FCS) 1% glutamine, and antibiotics. Proliferative responses were measured in triplicates of 200 μL containing 1 × 10⁶ total or CD3-depleted PBMC using various combinations of stimulating agents: immobilized anti-CD3 MoAb (60 ng/mL; OKT3); mitogenic combination of anti-CD2 MoAbs OKT11 and OKT11 (1:1000; kindly provided by Dr F. Malavasi, University of Turin, Turin, Italy); 1.2 μg/mL phytohemagglutinin (PHA; Irvine Scientific, Santa Ana, CA); 0.6 μg/mL pokeweed mitogen (PWM; Gibco); 5 ng/mL phorbol myristate acetate (PMA; Sigma). Data were acquired and stored in list mode. After activation (16 hours for CMOL and 96 hours for CD70 expression), cells were harvested, washed two times with PBS-FCS and incubated with PE-conjugated Streptavidin (Sigma).

After two washes in PBS-FCS, samples were analyzed by a flow cytometer equipped with an argon-ion laser (488 nm; Cytoron Absolute; Ortho) using the ABS software. Gates were set on lymphoid cells as determined by forward and right-angle scatter properties; dead cells were excluded by setting an appropriate threshold trigger on the low forward light scatter parameter, and nonspecific staining was assessed using FITC-conjugated nonimmune mouse IgG (Coulter). Data were acquired and stored in list mode. Fluorescence analysis was performed on a log scale.

**Cytokine assays.** IL-4 and IFN-γ were measured by sandwich enzyme-linked immunosorbent assay (ELISA) as described. The mouse MoAb 8F12 and biotinylated MoAb 3H·4 were used for IL-4, MoAb 42-103 (kindly provided by Dr G. Delespesse, University of Montreal, Montreal, Quebec, Canada) and biotinylated KM-48 (Kyowa Medex, Tokyo, Japan) were used for IFN-γ determination. The sensitivities of the IL-4 and IFN-γ ELISAs were 30 and 50 pg/mL, respectively. The binding to the second antibody was detected by stepwise incubation with alkaline phosphatase-conjugated streptavidin (Tago 6567; Tago, Burlingame, CA) and 4-nitrophenyl phosphate disodium salt (Merck, Darmstadt, Germany) and detected by 405 nm in a Vmax kinetic microplate ELISA reader (Molecular Devices, Sunnyvale, CA).

The IL-2 activity was measured by (3H)Thymidine uptake of IL-2-dependent CTLL cells. Serial dilutions of rIL-2 (1 U biologic activity = 0.1 ng/mL) or IL-2 containing supernatants were incubated with 3 × 10⁶ CTLL cells in 200 μL for 24 hours, followed by a 6-hour pulse of 1 μCi/well of (3H)Thymidine. This bioassay detects 50 pg/mL of human rIL-2.

IL-5 was estimated with a commercial ELISA-Kit (Quantikine; R&D Systems, Minneapolis, MN) as recommended by the manufacturer.

**Purification of eosinophils.** Eosinophils were isolated from human blood by negative selection of granulocytes that do not express CD16 incorporating a magnetic cell separation system (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany), as previously described. The purity was 98% to 99% as determined by light microscopy and flow cytometry. Cells were washed and resuspended in complete culture medium to a density of 1 × 10⁶/mL.

**Eosinophil culture.** Eosinophils were cultured in complete medium with 5% serum from the HES patient or from a control individual in the presence or absence of an antihuman IL-5 MoAb (10 μg/mL; R&D Systems, Abingdon, UK) for 48 hours at 37°C, 5% CO₂, and 95% air in a humidified atmosphere.

**Determination of eosinophil death by flow cytometry.** The relative amounts of viable cells were determined by uptake of 1 mmol/L ethidium bromide (Becton Dickinson, Mountain View, CA) in dead cells by flow cytometry analysis.

**DNA analysis.** High molecular weight DNA extraction and purification from blood lymphocytes was performed as previously described in detail. DNA was digested with three restriction enzymes: EcoRI, HindIII, and BamHI. Restriction fragments were electrophoretically size-separated on gel, transferred to nitrocellulose membrane that was prehybridized, and hybridized with 32P-labeled DNA probes. The DNA probe used in T study was genomic probe of the cDNA of the human TCRβ chain second constant region gene (Cβ2, 0.5-kb fragment). Each gel included one lane containing size markers composed of a HindIII digestion of λ phage and a lane containing high molecular weight human placenta DNA digested with the same restriction enzymes corresponding to that of the other samples in the gel. Hybridization and washings were performed under stringent conditions, and autoradiographic exposures on Kodak X-AR x-ray film (Eastman Kodak, Rochester, NY) were performed for 1 day.

**RNA extraction and cDNA synthesis and amplification by poly-
merase chain reaction (PCR). Total RNA was prepared from CD3\(^{-}\)CD4\(^{+}\) and CD3\(^{-}\)CD4\(^{-}\) T-cell subsets by the guanidinium thiocyanate-phenol-chloroform method. One microgram of RNA was retrotranscribed in the first strand of the B-chain-specific complementary DNA (cDNA) using the RiboClone cDNA Synthesis System Kit (Promega Corp, Madison, WI) and a primer specific for TCRBC1 and TCRB2 genes (5′CCA TGC GCC ACC CTA CCT CTT GAG GGG CTG CCG 3′). A fraction of cDNA was subjected to enzymatic amplification using a second human TCRBC primer (βα 5′ CCC ACT GTG CAC CTC CTT CC 3′) and a TCRBV degenerated primer [Vβ/β 5′ T(CTT)(A/C/T)(CTT)(AGT)GAT(A/C)(A/G)(A/T)CA 3′] that was designed to amplify B-chain rearrangements containing virtually all the known human TCRBV genes. 25 The conditions generate products of about 430 bp. PCR conditions were performed under the following conditions: denaturation for 39 cycles at 93°C for 1 minute, annealing at 52°C for 1 minute, and extension at 72°C for 1 minute; the last cycle extension was performed at 72°C for 7 minutes. The specificity of the total amplified product was analyzed using a colorimetric method and biotinylated TCRBV-specific probes. 26

To prevent sample contaminations, several precautions were taken. All solutions were aliquoted and used only once and a negative control (PCR master mix with primers for TCRBC and TCRBV degenerated without cDNA template) was processed with TCR samples in each step of the experiments. In addition, each T-cell sample was amplified for the β-actin gene to assess the integrity of the mRNA extraction.

RESULTS

Lymphocyte surface marker analysis. Immunophenotypical studies showed the presence of a CD3\(^{-}\)TCR-CD4\(^{+}\) population (Table 1A). All CD4\(^{+}\) cells were CD2\(^{+}\)CD5\(^{+}\) and more than 90% were CD28\(^{-}\)CD57\(^{-}\)CD11b\(^{-}\)CD25\(^{-}\) (Table 1B). Moreover, all CD4\(^{+}\) lymphocytes present in the CD3-depleted population (ie, CD3\(^{-}\)CD4\(^{+}\) cells) were CD45RO\(^{+}\) (data not shown). Unstimulated CD3\(^{-}\)CD4\(^{+}\) did not express CD40 ligand or CD30, whereas CD70 was weakly expressed. After stimulation with PMA plus ionomycin, normal expression of CD40L and unusual higher expression of CD70 compared with CD4\(^{+}\) cells of eight normal controls (85% v 12.1% ± 6.6%) was found on these cells (Fig 1).

Proliferative response. Proliferative response of total patient PBMC after activation with PHA and PWM was similar to that of healthy control, whereas it was reduced after activation with CD3 and CD2 (Table 2). On the contrary, CD3\(^{-}\)CD4\(^{+}\) enriched PBMC were not able to respond to mitogen activating the cells through cross-linking of membrane receptors (notably PHA, CD3, and CD2), but proliferative response and expression of activation markers such as CD69 and CD25 was partially restored when the transmembrane protein kinase C (PKC) activator PMA was added to PHA or was used in combination with ionomycin.

In vitro cytokine secretion. Unstimulated PBMC and PBMC depleted of CD3\(^{+}\) cells secrete very low levels of IL-4, IL-5, IL-2, and IFN-γ, similar to the healthy control (data not shown).

As shown in Fig 2, unfractionated PBMC from the patient and healthy control produced similar levels of IL-4, IL-2, and IFN-γ after stimulation with PHA plus PMA. Removal of CD3\(^{+}\) cells from patients’ PBMC with anti-CD3 MoAb precoated magnetic beads abrogated the ability to produce IL-2 and IFN-γ, but did not modify significantly the levels of IL-4 production. Of note, PBMC from patients produced exceedingly high levels of IL-5 on stimulation with PMA plus PHA compared with the healthy control. Also, this production was only partially reduced after removal of CD3\(^{+}\) cells, thus suggesting that this was essentially performed by CD3\(^{-}\)CD4\(^{+}\) cells.

Serum eosinophil survival factor study. A bioassay of eosinophil in vitro survival showed that patient’s serum contained much higher amounts of eosinophil survival factors than that of a healthy control, indicating that the CD3-CD4\(^{+}\) cells indeed produced cytokines with antiapoptotic properties for eosinophils. A neutralizing antihuman L-5 MoAb partially blocked the effect of the patient’s serum (Fig 3).

TCR analysis. Southern blot analysis of the DNA extracted from both CD3\(^{+}\) and CD3\(^{-}\) purified T-cell populations showed a polyclonal TCRBV repertoire. Analysis performed by reverse transcription-polymerase chain reaction of mRNA extracted from both CD3\(^{+}\) and CD3\(^{-}\)CD4\(^{+}\) purified T-cell populations showed a polyclonal TCRBV repertoire.
HES, IL-5, AND CD3-CD4' T CELLS

Fig 1. Expression of CD40L and CD70 on purified CD3-CD4' lymphocytes of patient (left) and CD3-CD4' lymphocytes of normal control (right). CD4' cells were obtained by positive selection with magnetic beads preceded by negative selection with CD3-coated magnetic beads as far as for the patients' cells. The cells were resting or activated with PMA (5 ng/mL) + ionomycin (500 ng/mL). Dashed lines represent the fluorescence of cells stained with nonimmune mouse IgG. Data are presented as relative log fluorescence intensity.

TCRBV rearrangement in CD3' cells and no RNA transcription in the CD3'CD4' population (Fig 4); in this latter case, the detection of β-actin amplification product showed the integrity of the mRNA extraction and amplification procedure (data not shown).

DISCUSSION

We present a case of HES associated with the presence of a cell population with an unusual CD3-, TCR-, CD4' immunophenotype, without detectable TCRBV mRNA and clonal rearrangement of TCRB gene, which was able to produce large amounts of IL-5 after stimulation.

This cell population was made of mature T lymphocytes; it had normal lymphocyte morphology and expressed T-cell characteristic antigens (CD2, CD5, CD7, CD28, CD45RO), and, after in vitro stimulation, CD40L but not markers (TdT, CD1a) of immature T cells. However, these cells lacked surface CD3 and TCR expression. A phenotype similar to that observed is quite frequent in peripheral T-cell lymphoma, being detected in 10% to 15% of cases.12,30 In our study, MoAbs directed against two different epitopes of the CD3 molecule were used to exclude the possibility that lack of recognition of the CD3 antigen was caused by genetic polymorphism; moreover, the lack of the CD3 molecule is concordant with the lack of reaction with MoAbs directed against the TCRAB and TCRGD frameworks.

A further remarkable phenotypic feature of this CD3'CD4' lymphocyte population was the expression (constitutively with low density, strongly positively modulated after stimulation with PMA plus ionomycin) of CD70, a molecule involved in the regulation of T-cell proliferation and survival.31 CD70 is not expressed on normal resting CD4' cells and only marginally after stimulation (personal observations).

Table 2. Proliferation Studies

<table>
<thead>
<tr>
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<th>Patient (PBMC)</th>
<th>Patient (PBMC/CD3')</th>
<th>Healthy Control (PBMC)</th>
</tr>
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<tbody>
<tr>
<td>Medium</td>
<td>770 ± 9</td>
<td>700 ± 95</td>
<td>1,230 ± 140</td>
</tr>
<tr>
<td>PHA (1.2 mg/mL)</td>
<td>72,850 ± 1,050</td>
<td>1,050 ± 35</td>
<td>115,150 ± 3,200</td>
</tr>
<tr>
<td>CD3 (60 ng/mL)</td>
<td>13,300 ± 270</td>
<td>700 ± 12</td>
<td>58,450 ± 1,600</td>
</tr>
<tr>
<td>CD2 (1-1,000)</td>
<td>18,650 ± 680</td>
<td>300 ± 7</td>
<td>48,550 ± 920</td>
</tr>
<tr>
<td>PWM (0.6 mg/mL)</td>
<td>20,700 ± 920</td>
<td>ND</td>
<td>25,400 ± 920</td>
</tr>
<tr>
<td>PMA (5 ng/mL)</td>
<td>± ionomycin (500 ng/mL)</td>
<td>15,150 ± 640</td>
<td>5,700 ± 120</td>
</tr>
<tr>
<td>PHA (1.2 mg/mL) + PMA (5 ng/mL)</td>
<td>ND</td>
<td>5,400 ± 150</td>
<td>ND</td>
</tr>
</tbody>
</table>

Proliferation studies of patients' total and CD3-CD4' enriched PBMC. Data are expressed as the mean cpm ± SEM of a triplicate culture.

Abbreviation: ND, not determined.
Despite its phenotypic abnormalities, there was no evidence of clonality by Southern analysis of TCRB gene rearrangement. This was performed using three different restriction enzymes, making it unlikely that rearranged bands were missed because of comigration of rearranged DNA fragments. The detection of germline bands can be explained by the presence of normal non-T cells in the population studied (whole PBMC preparation).

Deletion of TCRB gene in an abnormal (clonal?) population would better explain the lack of TCRB gene rearrangement detection as well as the lack of mRNA transcription and of the surface expression of its protein product, which were shown in the present case. Indeed, the absence of clonal TCRB gene rearrangement has been already described in several cases of CD3+ peripheral T-cell lymphomas and deletion of the gene has been suggested as a possible explanation. On the other hand, it is unlikely that these lymphocytes were of reactive nature. In fact, a stimulus should be hypothesized that is able to induce simultaneously in multiple T-cell clones a failure of TCRBV mRNA transcription and therefore the absence of membrane expression of TCRAB (and CD3). No normal population with these features has been described. We therefore believe that the abnormal lymphocyte population of this patient was the expression of a peripheral T-cell lymphoma with a very indolent clinical course.

Interestingly, lymphocytes with the same unusual phenotype were described in patients in whom the clinical onset of T-cell lymphoma was preceded by a several-year history of eosinophilia and in a recently reported case of eosinophilia, and in a recently reported case of eosinophilia,
in which the abnormal lymphocytes had a typical Th2 cytokine secretion pattern, were clonal, but were not clinically lymphomatous. This case was described as the first example of a proliferation of Th2 lymphocytes in humans.

Also in the present case, evaluation of in vitro cytokine production suggested that the abnormal T lymphocytes were responsible for the eosinophilia through the production of IL-5. This was supported by the demonstration of eosinophil survival factors in the patients' serum, whose activity was partially neutralized by an antihuman IL-5 antibody.

Their cytokine secretion pattern suggested that they belonged to the Th2 helper lymphocyte subgroup because they did not secrete either IL-2 or IFN-γ. Unlike the case reported by Cogan et al., our patient's cells did not produce high levels of IL-4, in accordance with the low serum IgE level of the patient. This feature did not preclude the assignment of these cells to the Th2 lymphocyte subgroup. Indeed, it has been recognized that the pattern of cytokine secreted by human T-cell clones may be more complex than that of a pure Th1/Th2 profile, and it seems that different environmental factors could be responsible for the functional polarization of T-cell clones towards a particular behavior. Although common mechanisms in the control expression of IL-4 and IL-5 have been shown, the possibility of a differential regulation has also been reported (e.g., IL-2 induces IL-5 but not IL-4 mRNA expression in mouse T cells). In vivo, a dissociation between IL-4 and IL-5 secretion was reported in nonatopic asthmatic patients. The lack of TCR/CD3 complex may further explain, in our study, this dissociation, as suggested by the ability of cyclosporin to inhibit IL-4 but not IL-5 CD3-induced production.

Another peculiar feature of this case was the absence of spontaneous in vitro secretion of IL-5, suggesting that persistent cell activation by an as yet unknown stimulus was responsible for its in vivo production. Although the CD3+CD4+ lymphocytes were not able to respond to signals acting through the TCR/CD3 complex, they expressed different structures (such as CD2 and CD28) related to the transduction of T-cell activating signals. Studies are in progress to see if the abnormal expression of CD70 by these cells could also play a role in this context.

In conclusion, we believe that this is a case of HES pathogenetically linked to the presence of an abnormal population of Th2 helper cells, which was able to selectively secrete large amounts of IL-5. From a clinical standpoint we will carefully observe the patient because a malignant disease may appear several years after the recognition of eosinophilia.

Moreover, because in this case the absolute lymphocyte count was within normal range, we suggest that a lymphocyte immunophenotypic analysis should be included in the diagnostic workup of every case of HES to detect the presence of even small subpopulations with an abnormal phenotype. Future studies should determine whether there is a specific association between eosinophilia and the CD3+CD4+ phenotype of lymphocytes, as suggested by this and several other reports.


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A case of hypereosinophilic syndrome is associated with the expansion of a CD3-CD4+ T-cell population able to secrete large amounts of interleukin-5

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