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ARTICLE

New diagnostic tool for differentiation of idiopathic hypereosinophilic syndrome (HES) and secondary eosinophilic states

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The hypereosinophilic syndrome (HES) is a very rare disease, characterized by persistent eosinophilia with tissue involvement and organ dysfunction which often precedes a subsequent T cell lymphoma. Interleukin-5 secreted by a T lymphocyte subpopulation has been described in previous reports as the most important factor responsible for the prolonged lifespan of the eosinophils. The goal of the present study was to describe a fast, simple diagnostic method for the differentiation of HES and secondary eosinophilic states. Beside the surface marker analysis of peripheral blood mononuclear cells (PBMC) we measured surface bound IgE molecules on lymphocytes and eosinophil cells, intracellular cytokines (IL-5, INFγ) in CD4+ lymphocytes and eosinophil major basic protein (MBP) in eosinophils using flow cytometric detection method. The appearance of an IL-5 producing cell population with a decreased number of INFγ positive lymphocytes was characteristic for the blood samples of HES patients. Predominance of Th2 cells with the appearance of a CD8+/CD3–/CD56+ cell population was restricted for the HES cases and could not be detected in secondary eosinophilic individuals. Our flow cytometric cytokine detection method (with parallel cell surface marker analysis) does not require cell separation or long term cell culture steps previously described for the detection of IL-5 producing cells. Therefore it seems to be a more appropriate approach for the differential diagnosis of primary and secondary eosinophilic states. (Pathology Oncology Research Vol 7, No 4, 292–297, 2001)

Keywords: hypereosinophilic syndrome, IL-5, INFγ, Th1-Th2 balance, flow cytometry

Introduction

High blood eosinophil counts in humans are detected in approximately 10% of the population, mainly due to parasitic infections, allergic processes or malignant diseases.[2,28,30] Distinguishing these from the very rare, but more serious idiopathic hypereosinophilic syndrome (HES) is a difficult task, sometimes prolonged diagnostic procedures are required.[1,29] HES comprises a heterogeneous group of disorders of unknown origin, where the usual causes triggering eosinophilia e.g. allergy, parasitic infection, autoimmune disorder can be excluded. It can be characterized by persistent eosinophilia in peripheral blood, bone marrow and eosinophilic infiltrates of multiple organs leading to severe organ dysfunction.[8,18] At present there is no diagnostic method available for the fast and precise distinction of idiopathic and secondary eosinophilic states.[12,14] The connection between hypereosinophilia and T lymphocytes with an aberrant phenotype is well established[13,17,23] manifested by the production of eosinophilopoietic cytokines.[21,25] CD4+ lymphocytes are the most important source of granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3) and IL-5 cytokines respectively, responsible for the development and differentiation of eosinophils in bone marrow.[6,26] The first two cytokines also stimulate the development of other bone marrow-derived cells, whereas IL-5 in humans acts more specifically on eosinophils.[22] Interestingly, IL-5 dramatically increases the life span of eosinophils by inhibiting their apoptotic cell death *in vitro.*[31] Similar effect of IL-5 detected in peripheral blood mononuclear cells would explain the persistent extreme eosinophilia in HES patients. The goal of the present study

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was, to find a diagnostic procedure suitable for the easy and reproducible differentiation of these conditions. Therefore we investigated the composition of the T cell pool (CD4/CD8, Th1/Th2 ratio) in peripheral blood samples of idiopathic and secondary eosinophilic patients, and measured their intracellular IL-5 and INFγ production by flow cytometry. An intracellular IL-5 positive cell population was detected among the CD4+ cells in HES patients, which was absent in secondary eosinophilic individuals. In parallel the frequency of INFγ producing cells decreased in HES, which might indicate a Th2 dominance. In contrast CD8+ T cell pre-dominance, and a characteristic CD3-CD8+ cell group (6-8%) appeared in HES patients, which proved to be CD56+ in triple labeling measurements. The presence of the CD3-CD8+CD56+ cell population and the IL-5 producing CD4+ cell group seem to be characteristic for the HES blood samples and could not be detected in secondary eosinophilic and healthy individuals.

Material and Methods

Patients

Fresh heparinized blood samples of 5 HES patients, 8 patients with secondary eosinophilia and 8 healthy (without any hematological or other disease) controls were obtained for flow cytometric analysis. The clinical diagnosis of HES was based on the diagnostic criteria of Chusid et al[5]. The clinical data of HES patients can be found in *Table 1.* The clonality examinations of T cells (TcR gene rearrangement) have been performed according to the method of Trainor et al.[27] The mean age of the secondary eosinophilic patients was 42.5 ± 14.4 years, their sex distribution was f/m = 1:1, their mean eosinophil counts before treatment were 4.8 ± 2.3 G/l. The diagnosis was toxocariasis in 2 patients, bronchial asthma in 2, chronic myeloproliferative disease and Hodgkin's disease in 1-1 patient, eosinophil gastroenteritis in 1 and trichinellosis in 1 patient. The age of the control group was 40.4 ± 8.6 years, the sex distribution was f/m = 1:1.

Materials

Phorbol myristate acetate, ionomycin, the protein transport inhibitor monensin, and all the tissue culture reagents were obtained form Sigma Chemical Co.(USA). The monoclonal antibodies used for cell surface labeling were purchased from DAKO A/S.

Detection of cell surface markers

Whole blood samples (collected in heparin) were stained with direct fluorochrome labeled mAbs (single, double and triple labeling), the erythrocytes were lysed (Becton Dickinson lysing solution) and the remaining cells were fixed (in 1% buffered formaldehyde) for flow cytometric analysis. FITC-labeled anti-human CD3, CD4, CD8, CD56, IgE, and isotype control IgG1 mAbs were used for single labeling. For double staining PE labeled anti-human CD4, CD8 or CD19 and isotype control IgG1 mAbs were used together with the above mAbs. For triple staining, we used a PECy5 labeled anti-CD3, anti-CD4-PE or CD8-PE and CD56 FITC antibodies.

Detection of intracellular eosinophil major basic protein (MBP)

Mouse anti-human eosinophil major basic protein (MBP) monoclonal antibody (Pharmingen Cat. No. 15381A) and FITC labeled anti-mouse IgG (SIGMA) was used for indirect staining of eosinophil cells[11] for flow cytometric analysis. Intracellular staining was performed as previously described[3], briefly 100 μl heparinized whole blood sample was used, lysed with lysis buffer (Becton Dickinson) then fixed with 4% paraformaldehyde for 20 min. After two washing steps in washing buffer (PBS, containing 0.1% BSA, 0.1% NaN_3) the cells were incubated with the appropriate dilutions of a-MBP mAb/or mouse IgG1 isotype control antibody in permeabilization buffer (PBS, containing 0.1% saponin,

Table 1. **Clinical data of HES patients**

	Age/sex	Year of diagnosis	Blood eo. (x 10^9/l)	Eo% BM	Infiltration	Therapy	BCRL/ABL	TcR clonality
1.	16/M	1995.	46,8	90%	Skin, kidney, heart, lung, gut	Steroid, IFN, hydroxiurea	Negative	N.D.
2.	44/M	1995	8.5	40%	Central nervous system	Steroid, hydroxiurea, BMT	Negative	Positive
3.	54/F	1999	3.3	40%	Lung, liver	Steroid	Negative	Positive
4.	47/F	1997	4,6	25%	Lung	Steroid	Negative	Positive
5.	69/F	1993	9,7	28%	Lung, liver	Steroid	Negative	Negative

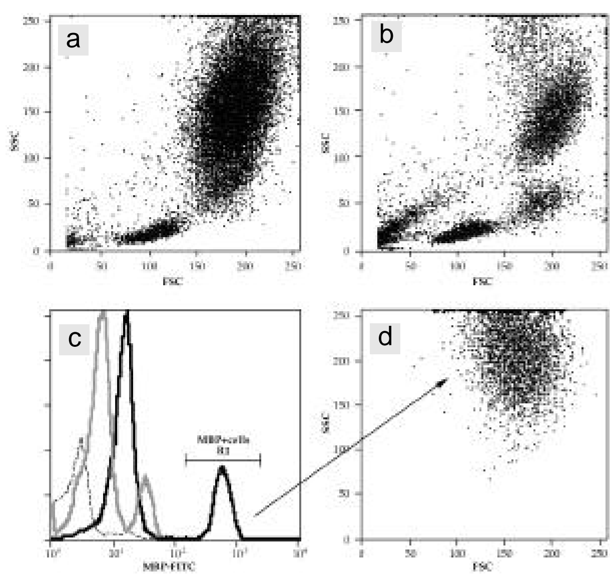

Figure 1. Characteristic white blood cell distribution was observed in peripheral blood samples of idiopathic (HES) ***(a)****, and secondary eosinophilic cases* ***(b)****. Well-defined cell populations could be distinguished in secondary eosinophilia, while in HES patients, the eosinophil and neutrophil cell population appeared as a confluent cell group. Flow cytometric measurement of intracellular eosinophil major basic protein (MBP) was used to distinguish eosinophils. Indirect MBP labeling (black curve) was compared to anti-mouse Ig-FITC (gray line) and autofluorescence samples (dotted line), represented on the histogram* ***(c)****. The MBP positive cells of the histogram (in R1 region) were gated and visualized on the FSC/SSC dot-plot* ***(d)****.*

0.1% BSA, 0.1% NaN_3) for 30 min. After two washing steps in permeabilization buffer anti-mouse IgG-FITC antibody was added for further 30 min. Then the cells were washed two times in permeabilization buffer and once in washing buffer, and the samples were resuspended in washing buffer for flow cytometric detection.

Stimulation of blood samples

Whole blood samples or peripheral blood mononuclear cells (PBMC) isolated on FicollPaque gradient (Pharmacia), were stimulated for 10-12 hours prior intracellular staining. The stimulation was carried out on sterile 24 well plates. Briefly 900 µl DMEM with 10% FCS (GIBCO), containing 15 ng/ml PMA + 1 µM ionomycin and 2 µM monensin was added to 100 µl whole blood samples (or 10^6 PBMC), mixed and incubated in 5% CO_2 at 37°C for 8-12 hours. After collecting the samples and washing in buffer (PBS containing 0.1% BSA, 0.1% NaN_3) the cells were labeled with cell surface marker antibodies (anti-CD4-PE or CD8-PE), then the red blood cell were lysed as described above.

Intracellular IL-5 and INFγ staining

FITC labeled rat anti-human IL-5 monoclonal antibody (PharMingen Cat.# 18054A) and anti-human-INFγ-FITC mAb (Immunotech Inc. France) were used for intracellular cytokine detection. After cell surface labeling, the samples were fixed in 4% paraformaldehyde (PFA) for 20 minutes, washed in washing buffer and incubated with the appropriate dilutions of a-IL-5-FITC or a-INFγ-FITC mAb/or mouse IgG1-FITC isotype control antibody in permeabilization buffer for 30 min. After two washing steps in permeabilization buffer and one washing step in washing buffer, the samples were resuspended in washing buffer and measured.

Flow cytometry

The samples were run on a FacsCalibur flow cytometer (Becton Dickinson, San Jose, CA). Data were collected for 10,000 events and analyzed using the CellQuest software. Electronic gating of FSC (forward scatter) versus SSC (side scatter) was used to eliminate cellular fragments and debris from different cell populations. Isotype-matched antibodies were used as negative controls. The specific cytokine staining in lymphocyte subpopulations was detected in samples stained both with PE labeled antibodies specific for surface markers and FITC labeled anti-cytokine antibody using an electronic gate combining their physical parameters and FL-2 staining, where FL-2 refers to the subset designations. Fluorescence histograms (in FL-1 channel) were created from this gate and the percentage of positive cells was statistically analyzed using the Cell Quest software.

Results

Flow cytometric detection of eosinophils

The cell surface marker analysis of peripheral blood samples of 13 eosinophilic patients and 8 healthy controls were examined and compared. According to the diagnostic criteria of Chusid et al,[5] the clinical diagnosis of 5 patients was HES (***Table 1***), while the remaining 8 were diagnosed with secondary eosinophilia. Characteristic distribution of white blood cells isolated from whole blood samples of eosinophilic patients (after haemolysis of red blood cells) could be detected by flow cytometry. In the forward scatter and side scatter dot plots, a characteristic group of highly granulated, large cells appeared more or less separate from the neutrophil granulocyte group. The size of this cell population corresponded to the eosinophil cell number of the patients. In HES cases this cell group was often found confluent with the neutrophil cell population, whereas in the secondary eosinophilic cases this phenomenon was rather rare[15] (***Figure 1a,b***). Indirect immunfluorescent staining of eosinophil major basic protein was used to distinguish eosinophils from neutrophil granulocytes. Specific and selec-

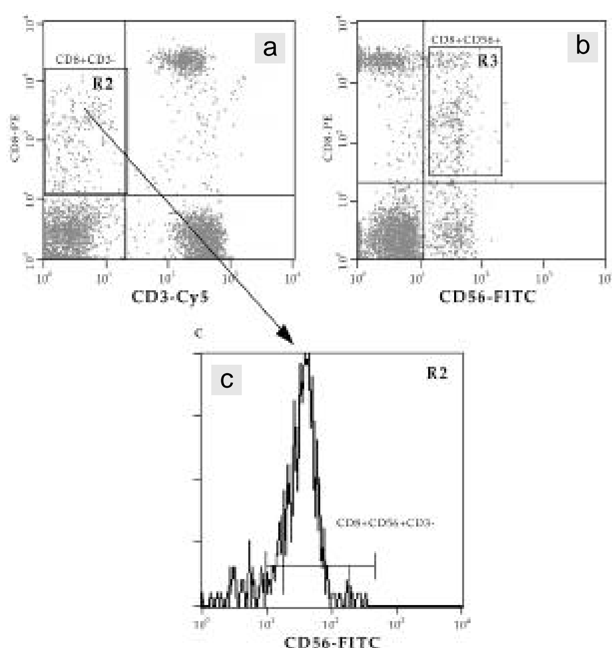

Figure 2. *A CD3-/CD8+/CD56+ cell group (in the lymphocyte gate) was detected in the blood samples of HES patients with triple labeling method and trivariate flow cytometric analysis. A CD8+/CD3- cell population (in R2)* ***(a)*** *and a CD8+/CD56+ cell group (R3)* ***(b)*** *were present on dual parameter dot plots. CD8+/CD3- cells gated in R2 were CD56 positive as well (FL-1 histogram)* ***(c).***

tive labeling of eosinophils was found both in secondary and idiopathic eosinophilic cases. The mAb did not stain neutrophils, monocytes or lymphocytes, thus making the selective identification of eosinophil cells possible (***Figure 1c,d***).

Lymphocyte surface marker analysis

Immunophenotypic studies of peripheral blood mononuclear cells showed characteristic differences between the HES and secondary eosinophilic cases. The CD4/CD8 ratio in the blood samples of HES patients was lower than that in secondary eosinophilic or healthy individuals. This increased CD8 cell population was characterized in double and triple labeling experiments. In the CD3/CD8 double stained samples of HES patients, a separate CD3-/CD8+ cell group appeared which represented around 6-10% of the lymphocytes. This cell population was further characterized, and in triple labeling experiments it constituted a subpopulation of CD3-/CD8+/CD56+ phenotype (***Figure 2***). These cells are present only in very low percentages in secondary eosinophilic and control cases (***Table 2***). In parallel we detected surface bound IgE molecules in secondary eosinophilic cases both on lymphocytes (8-10% of cells was positive) and on eosinophil granulocytes (100% positive). This was absent both in HES patients and healthy individuals (***Table 2***).

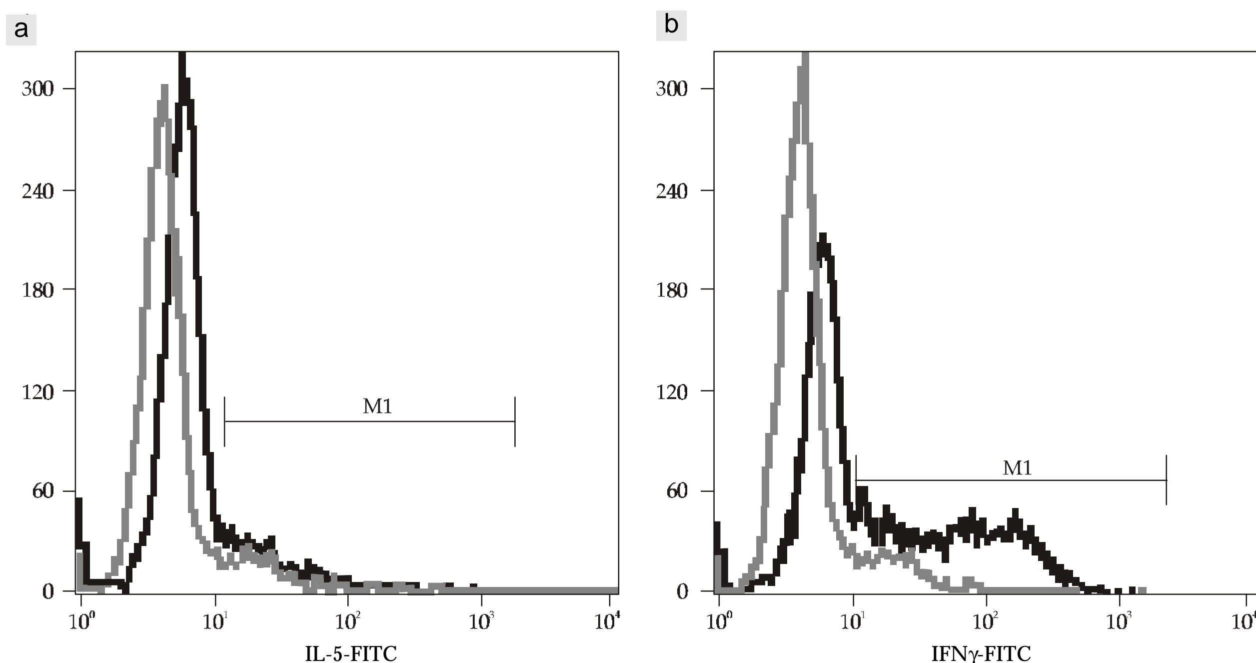

Figure 3. *Flow cytometric detection of intracellular IL-5 and INFg expression in control (only monensin treated) (gray line) and stimulated CD4+ cells (black line).* ***a)*** *Histogram of IL5-FITC fluorescence gated simultaneously on lymphocytes and CD4+ cells* ***b)*** *Histogram of INFγ-FITC fluorescence detected from the same gate. Percentage of marker positive (in M1) cells was determined in each sample and statistically analyzed.*

***Table 2.* Cellular composition of peripheral blood lymphocytes**

					Surface IgE+ %	
Samples	*CD3+%*	*CD4+%*	*CD8+%*	*CD3- /CD8+/CD56+%*	*Lympho*	*Eo*
HES patients *(n=5)*	66.2 ± 9.4	43.9 ± 8.2	27.5 ± 6.2	6.2 ± 1.8*	0.8 ± 0.2	Neg.
Sec. eosinophilia *(n=8)*	71.6 ± 6.3	51.5 ± 6.9	20.5 ± 6.7	1.4 ± 0.4	8.6 ± 0,8*	100
Control cases *(n=8)*	72.1 ± 9.5	48.8 ± 4.4	20.1 ± 5.8	1.8 ± 0.8	0.6 ± 0.1	Neg.

Results are expressed as percentage of positive cells in lymphocyte gate (mean ± SD).
The differences between HES patients and sec. eosinophilia were significant, *p<0.001

Detection of intracellular IL-5 and INFγ by flow cytometry

To detect the T helper cell polarization (Th1 – Th2) in our patients we measured the presence of IL-5 and INFγ producing CD4+ cells in the peripheral blood samples. In contrast to previous descriptions, where monoclonal antibodies (UCHT1, anti-human CD28) and recombinant cytokines were used for generating IL-5 producing cells (the whole Th2 population will secrete IL-5) in 2-4 day cultures, we used polyclonal activators in a short-term culture: phorbol esters together with calcium ionophore and monensin added simultaneously. In the blood samples of HES patients, 3-5% of the CD4+ cell population showed intracellular IL-5 expression after 8-12 hours activation. Without stimulation but in the presence of a protein-transport blocking agent (monensin) 1-3% of the cells were positive ***(Figure 3).*** On the other hand the fluorescence intensity increased in the result of activation and protein transport inhibition together. In secondary eosinophilic cases only 0.5-1.0 % of the CD4+ cells showed intracellular IL-5 staining after activation, which was significantly lower than in HES cases. Low or undetectable IL-5 level was observed in healthy controls (data not shown). In a parallel staining for INFγ production, reduced (13-15%) Th1 cell population could be observed in HES patients compared to the secondary eosinophil cases, where in contrast, 30-35% of the CD4+ lymphocytes showed INFγ production ***(Figure 4).*** This short-term activation allows us to measure the real in vivo conditions. This way we can detect the IL-5 producing cell population and not the whole Th2 cell group among the CD4+ Th cells.

Discussion

We present five cases of HES associated with the presence of an unusual CD3-/CD8+/CD56+ cell group and an IL-5 producing CD4+ cell population (3-5%) with the reduction of INFγ production in the peripheral blood. IL-5 is the most important cytokine determining the production and life span of eosinophils. Immunoserological detection of IL-5 in the serum of patients represents considerable technical difficulties due to the short lifetime and low concentration of this cytokine. Previous approaches included IL-5 measurements in the supernatant of isolated cell populations cultured in vitro[23] or IL-5 mRNA detection in purified PBMC and eosinophils.[21] Our goal was to find an IL-5 detection method that would be easily reproducible and useful for the diagnosis and distinction of secondary eosinophil and HES cases. Therefore we used intracellular flow cytometric methods for IL-5 and INFγ detection after short-term activation of PBMCs. The protocols described for intracellular IL-5 detection usually rely on long term (2-4 days) activation procedures using immobilized anti-human CD3 antibody together with soluble anti-human CD28 and external recombinant human IL-2 and IL-4. This stimulation is followed by adding PMA/ionomycin and protein transport inhibitor, during a further restimulation for 6 hr.[24] This stimulation method would reveal the total Th2 cell subset under circumstances that do not correspond to the real in vivo conditions. Our short-term activation has allowed us to measure the IL-5 producing cells (among the Th2 cells) that had been activated in vivo, prior

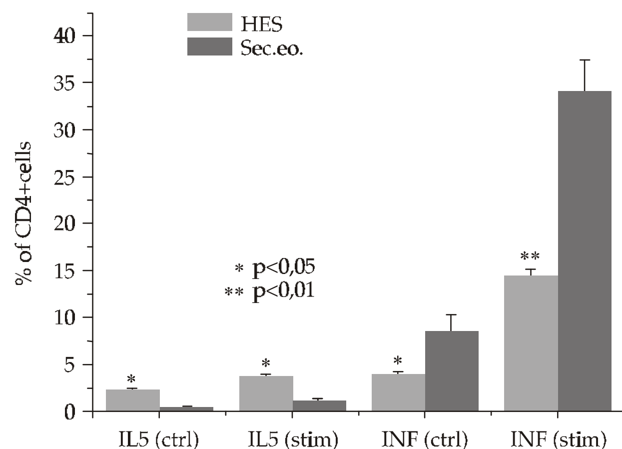

Figure 4. *IL5 and INFγ production of CD4+ cells was compared in HES (n=5) (gray columns) and secondary eosinophilic patients (n=8) (black columns). Peripheral blood samples of eosinophilic patients were treated with protein transport inhibitor (ctrl) or stimulated with PMA/ionomycin/monensin (stim.) as described in Materials and Methods. Results are expressed as mean ± SD.*

to their removal. The PMA/ionomycin/monensin activation increase the IL-5 production along with the simultaneous retention of IL-5 within the already committed Th2 cells, but will not generate new IL-5 producing cell populations in blood samples of HES patients. This way we have been able to detect an in vivo IL-5 producing CD4+ cell population without long term isolation and stimulation protocols. In parallel we found, that the physiological Th1 predominance was suppressed in these patients which could be followed by the lower number of the INFγ producing cells. Other reports have described an aberrant CD3-/CD4+ cell group[4] isolated from the peripheral blood sample of a HES patient (one case!), which can be induced to produce IL-5 ***in vitro.***[7] In our 5 HES cases, we have not been able to detect this aberrant Th2 cell group, however another unusual cell group of CD8+/CD3-/CD56+ phenotype appeared. This is presumably an abnormal T lymphocyte or natural killer cell subpopulation. Our finding support the common hypothesis that the presence of irregular T lymphocyte subsets[10,19,20] and their unregulated cytokine production may be the pathogenic factor in HES and a several-year history of HES precedes a subsequent T cell lymphoma.[9,16] Therefore patients with HES have to be observed carefully, because a malignant disease may appear several years after the recognition of eosinophilia.

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