The Abnormal Eosinophils Are Part of the Leukemic Cell Population in Acute Myelomonocytic Leukemia With Abnormal Eosinophils (AML M4Eo) and Carry the Pericentric Inversion 16: A Combination of May-Grünwald-Giemsa Staining and Fluorescence In Situ Hybridization

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The French-American-British subtype acute myelomonocytic leukemia with abnormal eosinophils (FAB AML M4Eo) with pericentric inversion of chromosome 16 is cytologically defined by a myelomonoblastic blast population and abnormal eosinophils. Until now, it remained an open question whether these abnormal eosinophils are part of the malignant clone or an epiphenomenon. We analyzed five cases of AML M4Eo with inv(16) and combined May-Grünwald-Giemsa staining with fluorescence in situ hybridization using yeast artificial chromosome clone 854E2, which spans the inv(16) breakpoint on 16p. In the case of inv(16), three instead of the normal two hybridization signals can be observed both on metaphase spreads and in interphase cells. With this approach, we were able to show inversion 16 in abnormal eosinophils and, therefore, identified them as a part of the leukemic cell population.

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ACUTE MYELOMONOCYTIC leukemia with abnormal eosinophils (French-American-British [FAB] classification AML M4Eo) is a distinct subtype of acute myeloid leukemias (AMLs) carrying specific morphological, cytogenetic, clinical, and prognostic features. The bone marrow (BM) morphology is characterized by a myelomonoblastic/monoblastic infiltration accompanied by a more or less significant fraction of abnormal eosinophils. They are detectable in the BM in nearly every case and sometimes can be found in the peripheral blood (PB) as well. These abnormal eosinophils show large, more irregular, deeply purple-staining granules, usually admixed with normal-appearing eosinophil granules. Abnormal eosinophils have distinct staining and cytochemical properties; in contrast to normal eosinophils, they are show large, more irregular, deeply purple-staining granules, usually admixed with normal-appearing eosinophil granules. Abnormal eosinophils have distinct staining and cytochemical properties; in contrast to normal eosinophils, they are active for naphthol-AS-D-chloroacetate-esterase staining and have a stronger reaction to periodic acid Schiff than do normal eosinophils. In 1982, Arthur and Bloomfield first reported on the association of BM eosinophilia and structural changes of chromosome 16. In 1983, Le Beau et al showed that a pericentric inversion inv(16)(p13q22) is consistently present in the AML M4 with abnormal marrow eosinophils. The association between morphology and cytogenetics is so strong that one can accurately predict the cytogenetic result of inv(16) in almost every case of AML M4Eo and vice versa. In 1985, this new subtype of AML was added to the revised FAB classification. Recently, breakpoint cloning identified the CBFB gene coding for the human homolog of a mouse transcription factor CBFB, also called PEBFQ and the MYH11 gene coding for a smooth muscle form of myosin heavy chain. The fusion transcript can now be detected by polymerase chain reaction (PCR). Furthermore, a yeast artificial chromosome (YAC) isolated during breakpoint cloning can be used for fluorescence in situ hybridization (FISH) in cases with inv(16)(p13q22). Until now, it remained unclear whether or not the abnormal eosinophils are part of the malignant cell population or are a result of a secondary response, an epiphenomenon. To answer this remaining question, we used a combination of standard May-Grünwald-Giemsa (MGG) staining and FISH to investigate abnormal eosinophils on the single-cell level and were able to show the inv(16) signals in abnormal eosinophils.

MATERIALS AND METHODS

Patients. Five cases of AML M4Eo were investigated. BM and PB smears were sent to our institution for central cytomorphology analysis, and the inv(16) was confirmed in all cases. Cytogenetic analysis was performed using standard techniques with G-banding. The description of the chromosome aberrations followed the rules of the International System for Human Cytogenetic Nomenclature. For the BM aspirate was isolated on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). DNA was extracted from the mononuclear cell fraction with a buffer containing guanidinium thiocyanate and phenol (RNAzol; WAK-Chemie, Bad Homburg, Germany) followed by chloroform purification. From the BM smear, DNA was dissolved directly on the glass slides with the RNAzol buffer. For both sources the RNA pellet was precipitated once in isopropanol and a second time in ethanol and afterwards was dissolved in 15 μL diethyl pyrocarbonate (DEPC)-treated water. The first-strand cDNA synthesis was performed with 7 μL of the RNA solution with avian myeloblastosis virus (AMV)-reverse transcriptase (cDNA-Kit Serva; Promega/Serva, Heidelberg, Germany) using random primers in a total reaction volume of 15 μL. The reaction was performed for 45 minutes at 42°C. A single-step PCR of the β-actin gene was used as a control for satisfactory sample cDNA. The CBFB/MYH11 fusion transcript was detected with the primers published by Claxton et al. PCR was performed with 1 μL of the cDNA product and Taq-polymerase (Boehringer, Mannheim, Germany) in a total reaction volume of 20 μL. After a denaturation step of 5 minutes at 94°C, 40 cycles of denaturation (15 seconds at 94°C), annealing (15 seconds at 59°C), and extension (15 seconds at 72°C) were performed, with a final extension time of 5 minutes. The PCR product was diluted 1:100 in water, and 1 μL of this solution was used for a second step of amplification with nested primers. The PCR products of the first and the second step were run on a 1.5% agarose gel, stained with ethidium bromide, and exposed under UV light.
Cytology. Air-dried and unfixed BM and PB smears were used for MGG and FISH studies. Investigations were made with fresh smears or after storage at -25°C. Some smears were stored unstained, whereas others were stained with MGG before freezing. The MGG staining was conducted according to standard procedures.

FISH. For FISH studies, the YAC 854E2 first described by Liu et al was obtained from Centre d’Étude du Polymorphisme Humain (CEPH; Paris, France). The probe was biotinylated by nick-translation using the BioNick kit (BRL, Rockville, MD) according to manufacturer’s instructions. This YAC binds to chromosome band 16p13 spanning the breakpoint involved in the inv(16)(p13q22). Therefore, in interphase cells showing a normal chromosome 16 and an inverted chromosome inv(16)(p13q22), we obtained in interphase cells with inv(l6) the hybridization signal is split and the cells contain three instead of two signals (Fig 1). In four cases, metaphases were additionally studied by FISH.

Combination of MGG and FISH. The combination of MGG staining and FISH was performed according to Anastasi et al with modifications. After MGG staining, the smears were covered with Pertex mounting medium (Medite, Wolfратхаuser, Germany), and morphological analysis was performed. Abnormal eosinophils were documented by conventional microphotography (Zeiss Axioskop, Jena, Germany). The location of the photographed cells on the smear was documented by the “mark and find system” (Zeiss). For FISH, coverslips were carefully removed after incubation in xylene for 5 minutes. Slides were fixed in Carnoy’s fixative (methanol:acetic acid, 3:1) for 15 minutes, washed in phosphate buffer for 1 minute, and fixed again in paraformaldehyde for 1 minute. After dehydration in ethanol (70%, 85%, 100%), FISH was performed according to a protocol published elsewhere. In brief, in situ hybridization was conducted as follows: 1 μL of hybridization mixture (0.5 ng biotinylated YAC DNA-probe, 100 μg human Cot-DNA, 10% dextran sulfate, 60% formamide, 1× SSC [0.15 mol/L NaCl and 0.015 mol/L sodiumcitrate, pH 7]) was placed on the area of the smear with the photographed cells, covered with a 10-mm round cover slip, and sealed with rubber cement. Slides and probes were denaturated simultaneously at 75°C for 3 minutes and hybridized at 37°C overnight. After hybridization, the slides were washed 3 times in 0.1× SSC at 60°C for 10 minutes each, followed by a brief equilibration in phosphate buffer at room temperature. The probe was detected by fluorescein isothiocyanate-conjugated avidin (Jackson/Dianova, Hamburg, Germany) followed by biotinylated goat anti-avidin-DN (Vector, Burlingame, CA). Amplification was performed by repeating both detection steps. Nuclei were counterstained by propidium iodide (0.5 ng; Sigma, Deisenhofen, Germany) and slides were covered with an antifade medium (90% [vol/vol] glycerine, 0.02 mol/L Tris HCL [pH 8], 2.3% [wt/vol] diazabicyclo[2.2.2] octane [DABCO], Sigma).

For evaluation of the signal constellation of the abnormal eosinophils, previously photographed cells were relocated by the “mark and find system” (Zeiss). The results were documented using conventional microphotography and the ISIS imaging system (MetaSystems, Sandhausen, Germany).

RESULTS

Cytomorphological studies. All cases were classified as AML M4Eo by standard cytomorphology. Abnormal eosinophils were detected in 3% to 25% in all BM specimens (mean, 15%; see Table 1 and Fig 2). Only in cases no. 2 and 5 were abnormal eosinophils observed in PB in 1% each.

Control studies for FISH. Control studies were performed on blood smears of three healthy volunteers. Constellations of FISH signals were investigated in a minimum of 200 interphase cells in each case. False-positive results, ie, three distinct signals in a cell, were obtained in 0.5%, 2%, and 5% of interphase nuclei, respectively. Therefore, the detection limit was defined at mean + 2 SD = 4.5%.

PCR. In all five cases, the fusion gene CBFB/MYH11 was detected using reverse transcriptase-PCR. Amplification with primers 3 and 2M produced a fragment of 280-bp according to transcript type A in all cases.

### Table 1. Clinical Data and Laboratory Results in Five Patients With AML M4Eo

<table>
<thead>
<tr>
<th>Patient Case No.</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Cytomorphological Diagnosis</th>
<th>Cytogenetic Result</th>
<th>CBFB/MYH11 PCR</th>
<th>% Blasts (MGG)</th>
<th>% Abnormal Eosinophils (MGG)</th>
<th>Interphases With inv(16) (FISH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>35</td>
<td>AML M4Eo</td>
<td>46,XY,inv(18)(p13q22) [10]</td>
<td>+</td>
<td>60</td>
<td>15</td>
<td>27% (27/100)</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>49</td>
<td>AML M4Eo</td>
<td>46,XY,inv(16)(p13q22) [17]</td>
<td>+</td>
<td>70</td>
<td>8</td>
<td>22% (68/300)</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>42</td>
<td>AML M4Eo</td>
<td>46,XY,inv(18)(p13q22) [16]</td>
<td>+</td>
<td>66</td>
<td>3</td>
<td>36.3% (109/300)</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>58</td>
<td>AML M4Eo</td>
<td>46,XY,inv(18)(p13q22) [16]</td>
<td>+</td>
<td>55</td>
<td>25</td>
<td>27.5% (55/200)</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>62</td>
<td>AML M4Eo</td>
<td>ND</td>
<td>+</td>
<td>43</td>
<td>15</td>
<td>26.5% (41/200)</td>
</tr>
</tbody>
</table>

Abbreviations: M, male; ND, not done.
ABNORMAL EOSINOPHILS IN AML M4Eo CARRY THE inv(16)

Fig 2. A case of AML M4Eo with abnormal eosinophils that show large, more irregular, deeply purple-staining granules and some eosinophils with normal-appearing granules (MGG; original magnification × 520).

Fig 3. The YAC 854E2 overstrained part of chromosome band 16p13 on the normal chromosome 16 and the inversion 16(p13q22) on the inverted chromosome 16 are shown. The interphase cell shows the signal constellation of inv(16)(p13q22) as well (case no. 3; original magnification × 320).

Fig 4. (A) The abnormal eosinophil in the BM showed the typical dysplastic irregular granules (case no. 5; MGG; original magnification × 720). (B) The abnormal eosinophil carries the inversion 16 with one signal of the normal chromosome 16 and one split signal of the inverted chromosome 16, as shown by FISH (original magnification × 540).
FISH studies. In three cases, metaphases could be analyzed after FISH. In 18 of 28 (64%) metaphases, a split signal was present on the aberrant chromosome 16 (Fig 3).

On BM smears, 100 to 300 interphase cells were investigated for the number of hybridization signals. The percentage of cells with the typical signal constellation of inv(16)(p13q22) ranged between 20.5% and 36.3% (mean, 27.5%; see Table 1).

In two cases, only one BM smear was available, and this was used for interphase FISH studies only; we were not able to combine MGG and FISH in these cases. In the other three cases, we combined the standard MGG staining with the FISH technique on blood or BM smears to investigate the signal constellation in abnormal eosinophils. Even where the hybridization signals were sometimes very weak in hypercellular areas on the BM smears, we were able to show that 56%, 63%, and 78% of the previously identified abnormal eosinophils contained three hybridization signals (Figs 4A and B).

DISCUSSION

AML M4Eo is a special subtype of AML and is characterized by a typical BM morphology and a pericentric inversion of chromosome 16. Abnormal eosinophils are detectable and define the FAB subtype AML M4Eo.3 They show irregular, deeply purple-staining granules and sometimes appear very dysplastic. The percentage of abnormal eosinophils differs between 1% and 45% in the BM. But even if only 1% of abnormal eosinophils are found in the BM, this should lead to the cytomorphological diagnosis of AML M4Eo.5,25

Abnormal eosinophils were described for the first time in 1969.3 They were characterized as an abnormal variant of the eosinophilic lineage and found to be positive for naphthol-AS-D-chloroacetate-esterase staining, in contrast to normal BM or blood eosinophils. Thus, a special subgroup of acute leukemias defined by this type of abnormal eosinophils was postulated.3,19 In 1983, Le Beau et al19 were able to correlate the cytomorphology of AML M4 with abnormal BM eosinophils with a pericentric inversion of chromosome 16 inv(16)(p13q22) in every case. This subgroup of AML was added to the FAB classification in 1985, and many studies in the last decade showed superior survival rates for patients with AML M4Eo and inv(16).3,6,20 Recently, YACs containing DNA sequences from the breakpoint on the short arm of chromosome 16 were isolated, and the fusion gene CBFB/MYH11 on 16p was identified at the cDNA level.14,16 Thus, FISH and PCR techniques became available for investigations in AML M4Eo.

Until now, it remained unclear whether or not the abnormal eosinophil is part of the malignant cell population.10,12,19 Recently, the state of the art was summarized by Liu et al in an excellent review.19 One study suggested the involvement of abnormal eosinophils in the malignant process by using metaphase analysis.27 Eosinophilic-stained granules were scattered beneath the metaphases with an inverted chromosome 16, leading to the assumption that the abnormal eosinophils belong to the aberrant clone. In another in vitro study, cells from the ME-1 cell line derived from a patient with AML M4Eo were induced to differentiate into eosinophils, which were shown to contain an inv(16).28,29 However, it is not clear whether these cells correspond to the abnormal eosinophils in vivo.

Thus, our starting point was the abnormal eosinophil detectable on routine BM or blood smears of five patients with AML M4Eo and inv(16)(p13q22). We combined the standard cytomorphological MGG staining with the FISH technique on air-dried smears. Using a "mark and find system" for every cell, we were able to prove that abnormal eosinophils carry the inversion 16 signals and, therefore, are clearly identified as a part of the leukemic cell population.

Given that specific probes are available for FISH analysis, this combination of cytomorphology and FISH obviously is useful for the detection of cell lineage-specific involvement in acute and chronic leukemias.30-33 It may also help to identify the origin of suspicious cells in the BM at the time of complete remission; therefore, it can be used for the detection of minimal residual disease.22,30,35-36

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The abnormal eosinophils are part of the leukemic cell population in acute myelomonocytic leukemia with abnormal eosinophils (AML M4Eo) and carry the pericentric inversion 16: a combination of May-Grunwald-Giemsa staining and fluorescence in situ hybridization

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