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Detection of Minimal Residual Disease in Acute Leukemia: Possibilities and Limitations*

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Abstract—*Studies are presented on the detection of 'minimal residual leukemia' using a monoclonal antibody (MCA) and fluorescence-activated cell sorting (FACS). As a preclinical model the BN rat acute myelocytic leukemia was used (BNML). The MCA Rm*124 *(IgM) strongly binds to BNML cells as measured by fluorescence intensity of a second-layer antibody (goat anti-mouse IgM fluorescein isothiocyanate). Only weak cross-reactivity occurred with normal mature granulocytes. It appeared possible to detect as low as* 1 *leukemic cell per* 10,000 *normal marrow cells, both in artificial mixtures and in marrows obtained after* in vivo *remission-induction chemotherapy with cyclophosphamide. Furthermore, an example is given of describing the kinetics of growth of leukemia in the liver, based on serial determinations of leukemic cells with the MCA technique. The population doubling time* (T_d) *in the liver calculated in this way did not significantly differ from that derived from time-consuming and expensive bioassays. Finally, the extrapolation of these techniques developed in a preclinical model to studies in human acute leukemia is discussed.*

INTRODUCTION

THE MAJOR problem in the present treatment of human acute leukemia is the maintenance of a complete remission. So far, no information is available on the number of residual leukemic cells in the bone marrow after successful remission induction chemotherapy. Although the minimal efficacy of this initial treatment can be expressed as a 2 log leukemic cell kill (based on classical cytological methodology) the actual reduction in tumor load per individual patient remains unknown. Starting with 10^{12} leukemic cells prior to therapy, the number left in the phase of 'complete remission' might thus vary between 0 and 10^{10} (Fig. 1).

As indicated in Fig. 1, two major approaches are presently being investigated for their efficacy to eradicate residual disease, i.e. (maintenance) chemotherapy and high-dose chemotherapy (e.g. cyclophosphamide) in combination with total-body irradiation (TBI) followed by bone marrow transplantation (BMT). If these methods fail to eliminate the clonogenic leukemic cells completely, leukemia relapse will occur. Decreasing the detection level as regards residual leukemia is essential for defining prognostic factors which lead to a more effective treatment strategy per individual patient. The changes in tumor load could then be quantified more accurately at various treatment stages and relapse could be predicted earlier. Furthermore, the total duration of treatment could be judged on a rational basis.

Several methods are presently being evaluated to detect 'minimal residual disease' (MRD; Table 1).

Besides chromosomal analysis, various discriminative concentration procedures are recognized. Furthermore, specific growth patterns of clonogenic leukemic cells may prove to be of value in detecting residual leukemia in bone marrow aspirates.

As regards monoclonal antibodies, various reports have appeared on monoclonals binding to (human) acute myelocytic leukemia (AML) cells [1-4]. However, all of these show cross-reactivity patterns with normal hemopoietic cells. This suggests that leukemia-associated specific antigens

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Table 1. Methods to detect residual leukemic cells

Technique	Leukemia-associated specific characteristic
1. Chromosome analysis	
a. cytometry	a. aneuploidy
b. premature chromosome condensation	b. proliferative-potential index
2. Concentration procedure	
a. monoclonal antibodies/FACS	a. specific antigens?
b. heterologous antibodies	b. malignant nucleolar antigens?
c. velocity sedimentation	c. cell size
d. density gradient centrifugation	d. cell density
e. free-flow cell electrophoresis	e. cell surface charge
f. combinations	
3. *In vitro* culture assays	specific growth pattern

do not exist. These antibodies rather detect differentiation-antigens linked to the myeloid lineage. If there are differences in the antigen density on the cell surface between normal and leukemic cells, leukemic cells might be specifically recognized by differences in antibody-labeling intensity, to be detected by fluorescence-activated cell sorting (FACS).

The present study describes the evaluation of a monoclonal antibody (Rm124) directed against the BN rat acute myelocytic leukemia (BNML) as regards the detection of small numbers of leukemic cells in (a) artificial mixtures of leukemic cells with an overload of normal marrow cells; (b) in the liver at various times after i.v. inoculation with leukemia; and (c) in bone marrow samples obtained at various times after remission-induction chemotherapy.

MATERIALS AND METHODS

The rat leukemia model

The BN acute myelocytic leukemia (BNML), which was induced with 9,10-dimethyl-1,2-benzanthracene in a female Brown Norway rat, shows striking similarities with human AML [5, 6]. Some of its major characteristics are: (a) a slow growth rate; (b) a severe suppression of normal hemopoiesis due to an absolute numerical decrease in the number of hemopoietic stem cells (CFU-S); (c) the presence of clonogenic leukemic cells (*in vivo*: LCFU-S; *in vitro*: clonogenic assays); (d) response to chemotherapy as in human AML. An additional advantage of this model is that normal stem cells (CFU-S) and leukemic clonogenic cells (LCFU-S) can be selectively discriminated by modified spleen colony assays [7].

The Rm124 monoclonal antibody

This monoclonal antibody (IgM) was produced and provided by Drs H. Kaizer and R. J. Johnson, Johns Hopkins University, Baltimore, MD, U.S.A.

Immunofluorescence labeling of cells

Bone marrow or liver suspensions were prepared with Hanks' HEPES-buffered balanced saline solution (H.HBSS). The cell suspension was centrifuged, resuspended in H.HBSS supplemented with inactivated fetal calf serum (FCS, Flow Lab.; 5% v/v) and sodium azide (0.01% v/v), which is used throughout the whole labeling procedure. For fluorescence labeling studies 10^6 cells were pelleted and labeled with the MCA-Rm124 (50 μl) at various dilutions at 0°C for 45 min. After careful washing the cells were incubated with a goat anti-mouse IgM (Fc) coupled to fluorescein isothiocyanate (GAM/IgM(Fc)/FITC 1/30) for 30 min at 0°C. After washing the cells were resuspended and processed on the FACS II cell sorter.

Light-activated cell sorter

After labeling the cells were analyzed on a modified FACS II (Beckton Dickinson, Sunnyvale, CA, U.S.A.) light-activated cell sorter with the laser beam at 488 nm (0.4 W). FITC fluorescence was measured through a combination of a broad-band multicavity interference filter (520–550 nm transmission, Pomfret, Stamford, CT, U.S.A.) and a 520-nm cut-off filter (Ditric) by an S-20 type photomultiplier. Perpendicular light scatter (PLS) intensity was measured by an S-11-type photomultiplier. PLS Signals were linearly amplified. A logarithmic amplifier (T. Nozaki, Stanford, CA, U.S.A.) was used for the fluorescence signals.

RESULTS

The fluorescence intensity profiles of normal rat bone marrow cells and BNML cells after

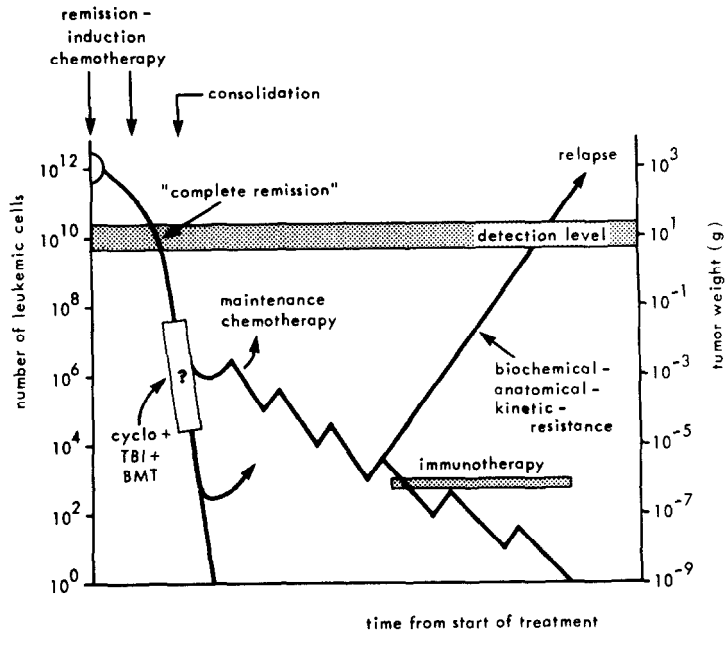

Fig. 1. Acute leukemia: principles of treatment.

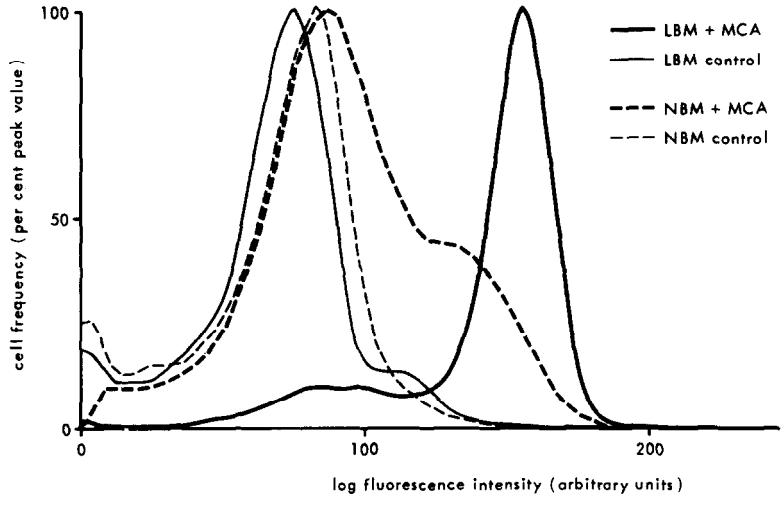

Fig. 2. Fluorescence intensity profiles of normal rat bone marrow and BN leukemia bone marrow after labeling with the Rm124 monoclonal antibody (MCA). LBM/NBM: leukemic/normal bone marrow cells.

labeling with the Rm124 monoclonal antibody (MCA) are shown in Fig. 2.

It is clear that the majority of leukemic cells are strongly positive. However, there is some overlap with a subpopulation of normal marrow cells. Upon sorting of the positive (normal) cells, they appear to be mainly mature granulocytes as judged by microscopic examination. Based on this cross-reactivity, it is suggested that the Rm124 MCA detects a differentiation antigen. From studies to be reported elsewhere it is clear that the MCA does not bind to normal hemopoietic stem cells [8].

In subsequent experiments various artificial mixtures of normal and leukemic marrow cells were incubated with Rm124 and thereafter with goat anti-mouse IgM fluorescein isothiocyanate (GAM-FITC). The fluorescence intensity profiles are shown in Fig. 3.

Up to a concentration of 1 leukemic cell per 10^4 normal marrow cells a small but distinct highly fluorescent subpopulation of cells is observed. It should be noted that data on the Y-axis are plotted logarithmically. After the fluorescent subpopulation of cells is sorted out, as is indicated for the 1/100 mixture (Fig. 4), 92% of the leukemic cells present originally are recovered. The remainder of the sorted positive cells are granulocytes and some promyelocytes, myelocytes and metamyelocytes.

In the liver, 3 days after i.v. leukemia transfer leukemic cells were detected with the MCA

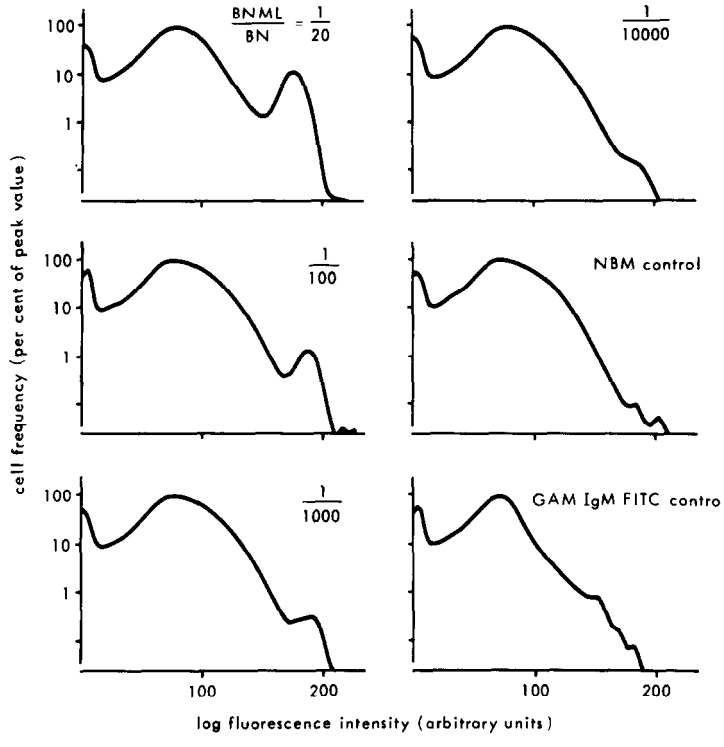

Fig. 3. Fluorescence intensity profiles of various mixtures of normal (BN) and leukemic (BNML) bone marrow cells after labeling with the MCA-Rm124 (BN acute myelocytic leukemia). NBM: normal bone marrow cells.

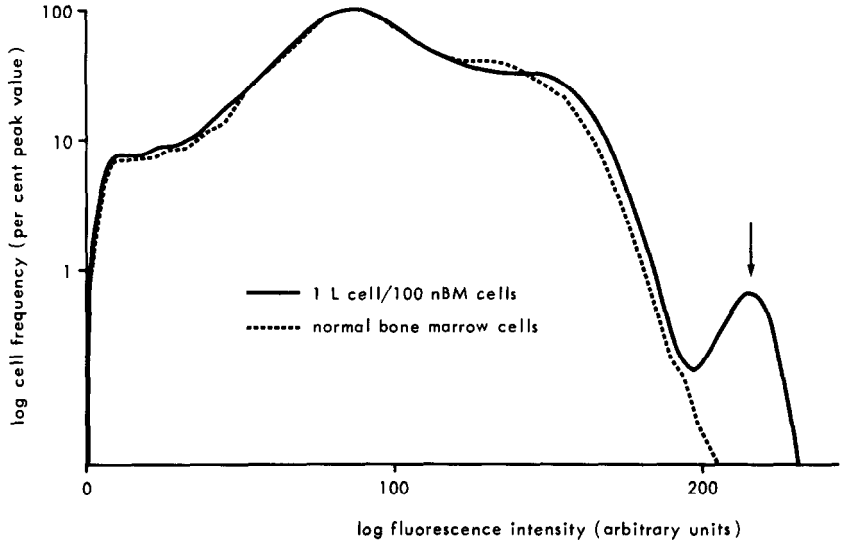

Fig. 4. Detection of minimal residual disease with a monoclonal antibody (Rm124) against the BN acute myelocytic leukemia. L cell: leukemic cell; nBM: normal bone marrow.

Rm124 (Fig. 5). The number of leukemic cells is at that time already far above the minimal detection level, i.e. 10^7 leukemic cells were scored by the FACS machine in a total number of 130×10^7 liver cells (1 per 130 cells). Serial determinations at various times after BNML inoculation yielded a growth curve of leukemia in the liver, characterized by a population doubling time (T_d) of 1.3 days (Fig. 5, upper curve). The growth kinetics derived in this way are not different from those obtained by conventional bioassays ($T_d = 1.2$ days; Fig. 5, lower curve). With the latter method the absolute number of leukemic cells present in the liver is deduced from the survival time of recipient rats injected with a fixed amount of that particular liver. As survival time is linearly correlated with the number of leukemic cells injected [5], this time-consuming and expensive method indirectly provides the data requested. The observation that both curves differ in terms of the time-tumor load relation is most likely due to the fact that in the MCA experiment only viable,

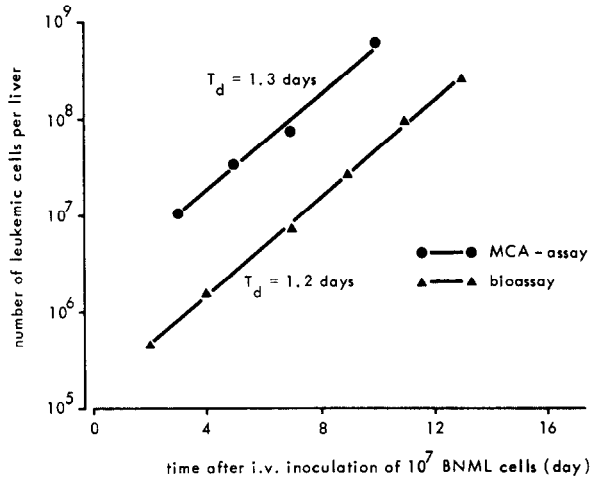

Fig. 5. Growth kinetics of the BNML in the liver. Comparison of direct measurements with monoclonal antibodies (MCA; Rm124) and in vivo bioassays (3–5 rats per point).

nycodenz-enriched leukemic cells were injected, in contrast to the bioassay, where 'total cells' (including dead cells) were used.

A more realistic approach to the detection of residual leukemic cells is offered in the next experiment. Rats were inoculated with 10^7 BNML cells. At day 13 after inoculation cyclophosphamide was injected i.p. in a dose of 100 mg/kg. This dose induces a 5 log leukemic cell kill [9]. As the total tumor load at day 13 is 5×10^9 cells, about 5×10^4 leukemic cells survive this treatment. The frequency of BNML cells in the femoral bone marrow at various times after cyclophosphamide is given in Table 2. This frequency was derived by injecting graded numbers of bone marrow cells into normal recipient BN rats. The survival time of these rats is linearly related to the number of leukemic cells present in the inoculum.

From Table 2 it appears that the frequency of leukemic cells among normal cells decreases till around day 22, i.e. till 9 days after cyclophosphamide treatment. This protracted virtual decrease is due to the regrowth of normal marrow which is faster than that of the leukemic cell population.

The fluorescence intensity profiles of marrow suspensions incubated with the Rm124 MCA at

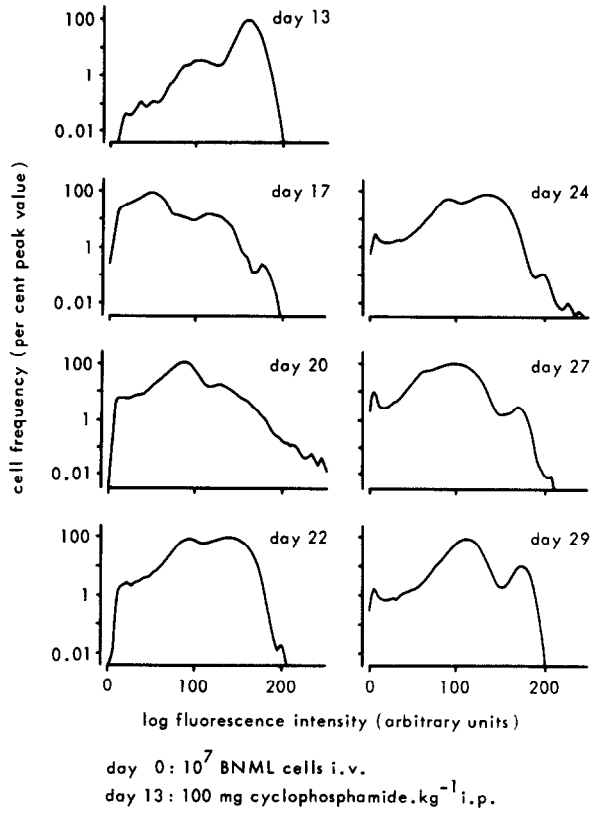

Fig. 6. Detection of residual leukemia in the femoral bone marrow with the Rm124 monoclonal antibody after high-dose cyclophosphamide tratment (BN acute myelocytic leukemia).

similar time intervals after chemotherapy are shown in Fig. 6.

Apart from day 20, where apparently an overload of cell debris and macrophages does not allow the detection of leukemic cells, a clear-cut highly fluorescent cell population is observed at the other sampling times. After sorting, the cells are mainly leukemic. Thus as low as 1 leukemic cell per 16,000 normal marrow cells (day 22, Table 2) can be detected with the monoclonal antibody approach.

DISCUSSION

Figure 7 summarizes the presently available methods to detect minimal residual disease in the BN acute myelocytic leukemia.

In general, dose survival bioassays offer the most sensitive method of detection in animal

Table 2. Detection of residual leukemic cells in the femoral bone marrow after remission-induction chemotherapy with cyclophosphamide

Days after 10^7 BNML cells i.v.	Days after cyclophosphamide	Frequency of BNML cells*
17	4	1/1000
20	7	1/3500
22	9	1/16000
24	11	1/7800

Cyclophosphamide: 100 mg/kg i.p. at day 13.
*Determined by survival bioassays.

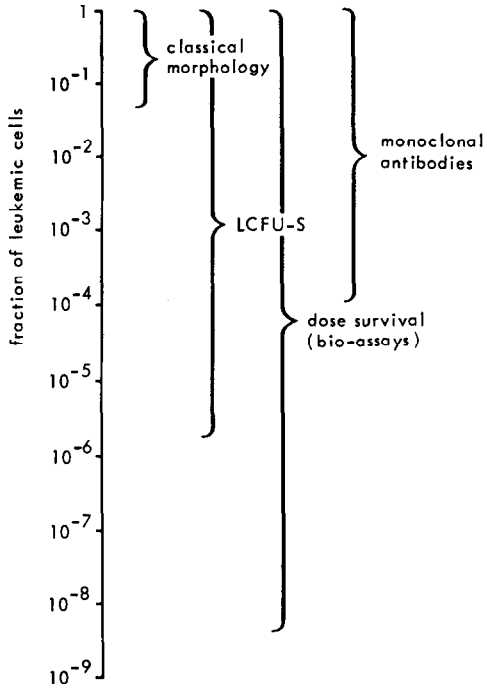

Fig. 7. Limitation of the available methods for the detection and quantification of minimal residual disease in acute leukemia (BN acute myelocytic leukemia).

model systems (8–9 log range). In the BNML this is followed by clonogenic leukemic cell assays (LCFU-S; 5–6 logs) and detection with monoclonal antibodies (4 logs). The MCA approach adds 2.5 logs to the detection level feasible with conventional cytological means.

These animal model studies are meant to provide a base for clinical explorations. In this respect, it is recognized that the clinical conditions are more complex. First of all, the presently available monoclonal antibodies against AML show quite some cross-reactivity with normal marrow cells [1–4]. Secondly, as only a relatively small fraction of the total marrow cellularity in man can be aspirated for diagnostic purposes and cell-sorting technology is still rather 'slow', the lower limit of detection of residual leukemia will be at the most in the order of 10^6 cells. This is illustrated in Fig. 8.

With the clinical approach it will be necessary to combine various methods of concentrating and identifying residual leukemic cells. It is envisaged to combine density gradient centrifugation with (double) MCA labeling procedures and

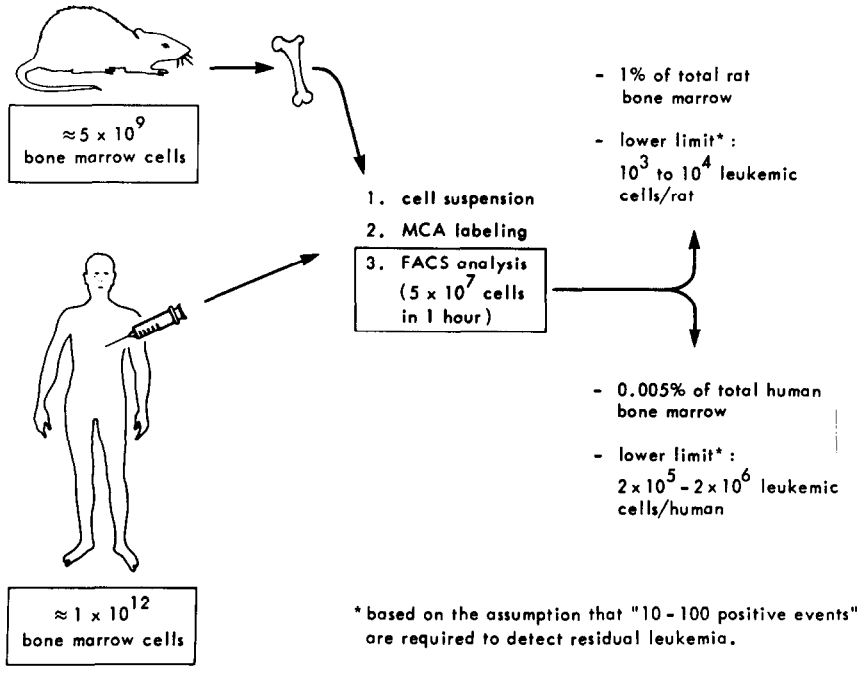

Fig. 8. Lower limits of detection of minimal residual disease in acute leukemia: rat vs man.

fluorescence-activated cell sorting. Further analysis on the sorted cell fraction should include immunoperoxidase stains, karyotyping and/or electron microscopy.

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