Dynamics of Precursor Cell Composition in Bone Marrow Culture Derived from Mice Deficient by Tumor Necrosis Factor

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Hemopoiesis in a long-term bone marrow culture derived from mice deficient for tumor necrosis factor was maintained for more than 130 weeks, which was 4 times longer than in cultures from wild type mice. The dynamics of hemopoietic precursor cells of different maturity was studied in the suspension fraction of the culture. The incidence of granulocyt-macrophage precursor cells and cell forming cobblestone areas was studied by the method of limiting dilutions in culture. In contrast to cultures derived from wild type mice, in long-term bone marrow culture derived from tumor necrosis factor deficient mice, first, the incidence of early precursor cells gradually increased and then the incidence of all precursor cells sharply increased.

Key Words: *stem hemopoietic cell; cells forming cobblestone areas; long-term bone marrow cell culture; mice deficient for tumor necrosis factor gene*

In long-term bone marrow culture (LTBMC) derived from wild type (WT) mice hemopoiesis ceases after 16-20 weeks, while in LTBMC derived from mice deficient by tumor necrosis factor ($TNF^{-/-}$) hemopoiesis is stable, and the oldest culture reached the age of 140 weeks, which is comparable with mouse life span. It was previously shown that long-term maintenance of hemopoiesis in culture was not due to changes in precursor cell proliferation and capacity to apoptosis [1]; this phenomenon was a result of neoplastic transformation of cells in $TNF^{-/-}$ LTBMC [2]. Quantitative analysis of precursor cells in $TNF^{-/-}$ LTBMC can explain principal changes in hemopoiesis in LTBMC.

In this study we counted the number of committed (granulocytic macrophagal) precursor cells (CFU-GM) and evaluated the profile of hemopoietic precursors by the number of cells forming the cobblestone areas (CFCA) [9]. The number of CFCA at different terms of culturing reflects the number of precursor cells of different degree of maturity. For example, the number of CFCA on day 7 (CFCA-7) correlates with the number of precursors forming colonies in the spleen of irradiated mice, while the number of CFCA on day 28 (CFCA-28) reflects the number of cells repopulating the bone marrow [8,9]. Simultaneous measurement of CFU-GM, CFCA-7, and CFCA-28 allows evaluation of the entire spectrum of hemopoietic precursor cells in LTBMC from primitive (CFCA-28) to committed (CFU-GM). Regular measurements of this spectrum in $TNF^{-/-}$ LTBMC show changes in the composition of precursor cells and the terms and type of these changes.

MATERIALS AND METHODS

C57Bl/6 male and female mice aged 10-28 weeks were obtained from the vivarium of Affiliated Department of Institute of Bioorganic Chemistry (Pushchino).

LTBMC were prepared using Dexter's method [3]. Bone marrow from one femur was transferred (without making single-cell suspension) to 25-cm^2 plastic flasks with 10 ml complete nutrient medium

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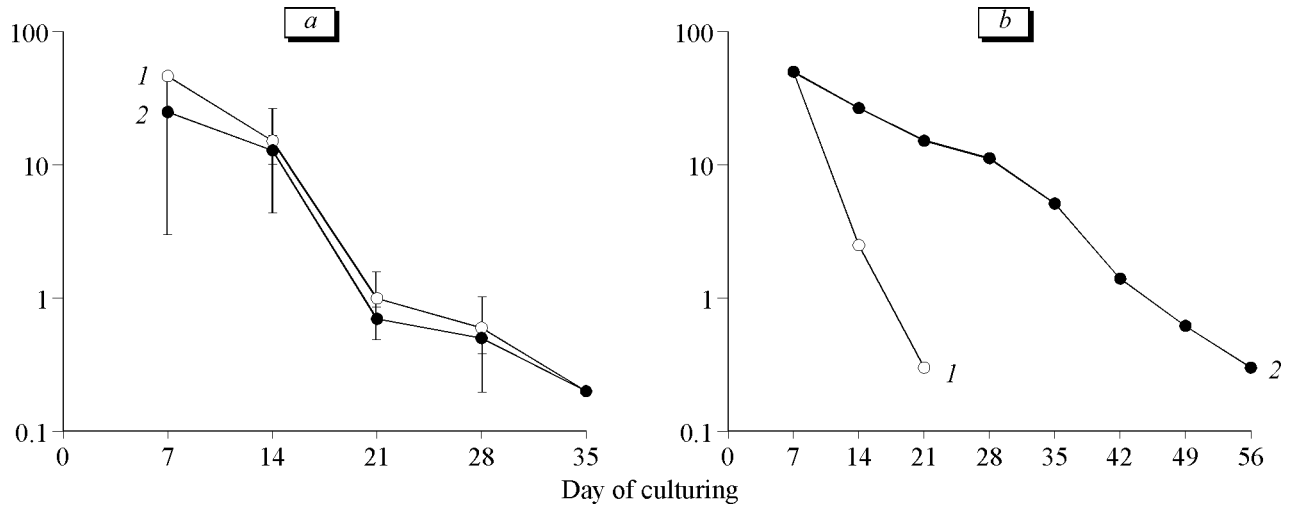

Fig. 1. Profiles of cells forming cobblestone areas in long-term bone marrow culture during weeks 3 (*a*) and 30 (*b*). The data of two experiments are averaged. *1*) wild type mouse cells; *2*) cells derived from tumor necrosis factor-deficient mice. Here and in Figs. 2 and 3: ordinates: incidence of cells per 10^5 suspension fraction cells.

(Fisher medium with glutamine, antibiotics, 10^{-6} M hydrocortisone, and 20% serum: $^1/_3$ fetal calf serum and $^2/_3$ equine serum). The medium was half-replaced once a week.

For evaluation of the number of CFCA, single-cell bone marrow suspension from two femoral bones of WT mice in Dexter medium was inoculated in 60 wells of a 96-well plate. For preventing drying and contamination, 200 µl 0.1 M NaOH was put into all edge wells. The plates were cultured at 33°C and 5% CO_2 in humid atmosphere. After 3 weeks the plates were irradiated on an IPK ^{137}Cs device (10 min, 435 sGy/min), the medium was replaced, and suspension fraction cells from WT and TNF$^{-/-}$ LTBMC were inoculated onto the resultant sublayer in 4 serial dilutions for estimating the number of CFCA by limiting dilutions analysis. As a rule, 50, 25, 12,5, and 6.25 thousands cells per well were inoculated, 15 wells per dilution. The medium was half-replaced once a week.

The wells were examined under an inverted microscope every week before replacing the medium. Negative (containing no CFCA) wells were counted. The probability of a CFCA-free well is described by the Poisson distribution: $P_0=e^{-m}$, where m is the mean number of CFCA in the sample. This probability was estimated as $P=r/n$, where n is the number of wells for the dilution and r number of negative wells for the same dilution, and hence $m=-\ln(r/n)$. The mean number of CFCA (m) in a sample can be estimated. Under our conditions the number of CFCA can be estimated in a range from 0.14 (1 positive well in dilution 50,000 cells/well) to 43.4 (1 negative well in dilution 6250 cells/well) per 100,000 inoculated cells.

The number of CFU-GM in semiliquid agar cultures was evaluated by the standard method [1].

The percentage of hemopoietic cells in the suspension fraction was evaluated using common leukocytic marker CD45 [5]. Suspension fraction cells were fixed with 4% paraformaldehyde and incubated (in succession) with rat antimurine antibodies to CD45 (BD Pharmingen) and goat anti-rat FITC-conjugated immunoglobulins (BD Pharmingen).

RESULTS

The incidence of CFCA in cultures of both types was evaluated every 3 weeks. After 3-week culturing the precursor spectra in LTBMC from WT and TNF$^{-/-}$ mice were similar (Fig. 1, *a*), while after 9 weeks the number of CFCA-28 in TNF$^{-/-}$ culture was higher than in WT culture. During week 21 appreciable differences in the number of more differentiated precursors (CFCA-7) in the two cultures were detected: their incidence was higher in TNF$^{-/-}$ culture. During week 30 the number of late precursors (CFCA-7) surpassed 50×10^5 suspension fraction cells in both TNF$^{-/-}$ and WT culture, *i.e.* it was beyond the sensitivity range of this method. Therefore, we cannot know whether the

TABLE 1. Immunophenotypical Characteristics of TNF$^{-/-}$ LTBMC ($M\pm m$)

Time of culturing, week	Cell phenotype, %		
	CD-45	Mac-1	Gr-1
25-27	98.7±0.4	99.8±0.1	61.5±9.5
67-69	4.2±1.3	1.9±0.8	0
103-105	5.9±0.9	0.8±0.45	0.2±0.1
142-144	9.2±2.1	3.8±1.6	0

number of CFCA-7 decreased in any culture by this term. However, the differences in the number of earlier precursors were obvious: WT culture contained no CFCA-28, while in TNF$^{-/-}$ culture their number was 11.2×10^5 cells (Fig. 1, *b*).

The number of CFCA-7 before week 18 was virtually the same in the two cultures (Fig. 2, *a*). At later terms, the number of CFCA decreased in WT LTBMC and after week 33 they cannot be detected, while in the TNF$^{-/-}$ LTBMC suspension fraction the number of CFCA markedly increased and by week 33 was beyond the sensitivity range of the method. A similar picture was observed for less differentiated precursors (CFCA-28, Fig. 2, *b*). Significant differences in their number in WT and TNF$^{-/-}$ LTBMC were observed starting from week 15, while after week 21 of culturing CFCA-28 were no longer detected in WT LTBMC, that is, their number was below the sensitivity threshold of the method. A drastic increase in the number of CFCA-28 in TNF$^{-/-}$ LTBMC was observed during week 33 of culturing, after which the number of precursors slightly fluctuated at a high level in TNF$^{-/-}$ cultures.

In TNF$^{-/-}$ cultures the number of more differentiated committed CFU-GM precursors increased simultaneously with the increase in the number of early precursors (Fig. 3). After 60 weeks of culturing nonhemopoietic cells were detected in the suspension fraction. The percentage of CD45-positive cells decreased throughout culturing (Table 1), while the percentage of cells carrying terminal differentiation markers, *e.g.* Mac-1 (characteristic of granulocytes and macrophages) [10] and Gr-1 (granulocyte marker) [4], decreased to 0-4%.

Our experiments showed important differences between WT and TNF$^{-/-}$ LTBMC. Hemopoiesis completely ceased in WT culture after 30 weeks of culturing, while in cultures expressing no TNF the number of precursor cells of different maturity drastically increased. The increase in the concentration of precursor cells was paralleled by a drop of their differentiation capacity in culture; however they retain the

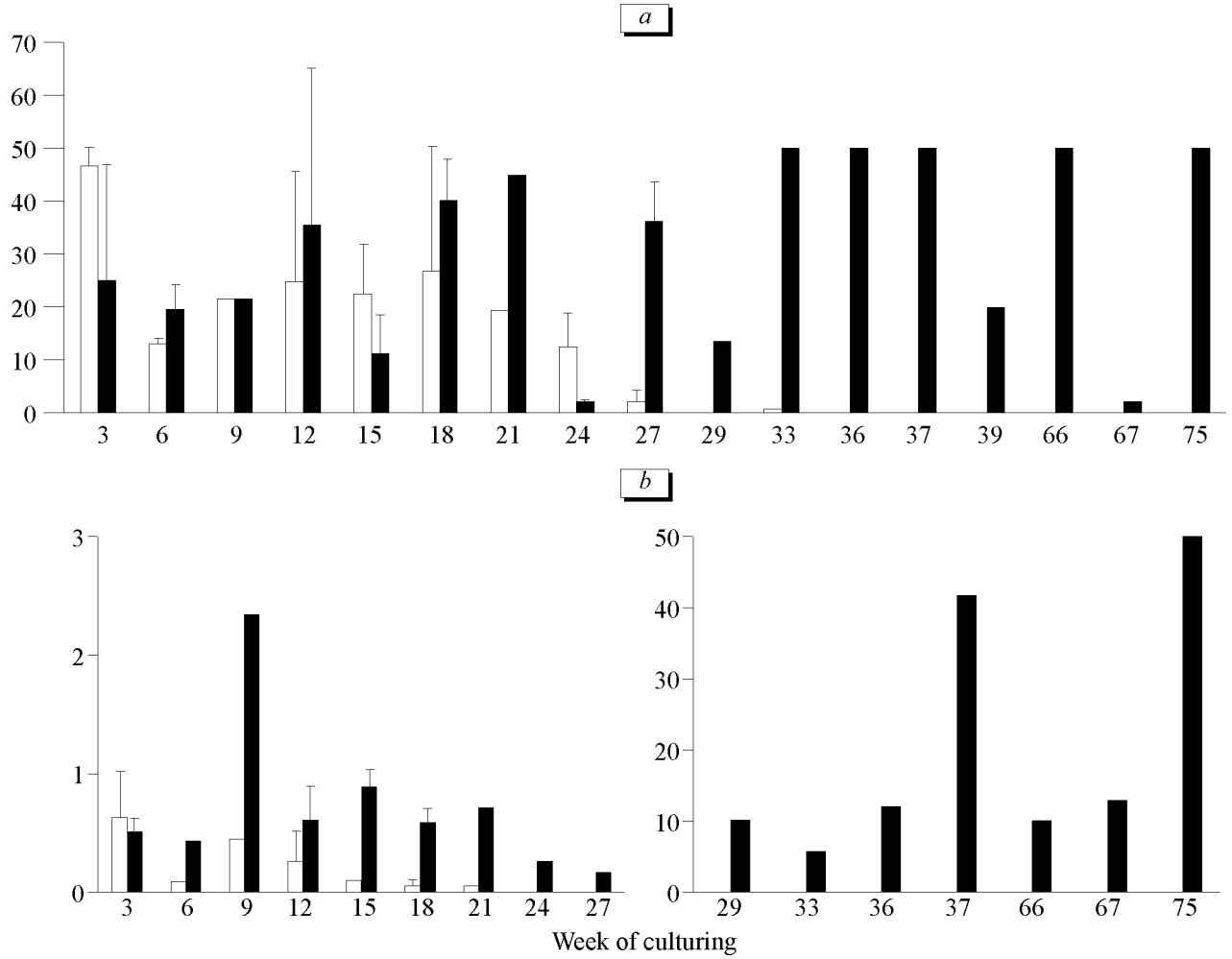

Fig. 2. Dynamics of cells forming cobblestone areas in the suspension fraction of long-term bone marrow culture derived from wild type mice (light bars) and mice deficient by tumor necrosis factor (dark bars). *a*) late non-committed precursors; *b*) the earliest precursor cells.

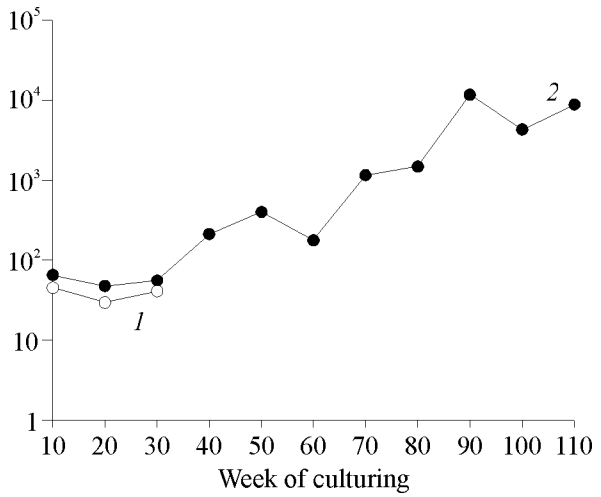

Fig. 3. Concentration of granulocyte-macrophage precursor cells in long-term bone marrow cells culture derived from wild type mice (*1*) and tumor necrosis factor-deficient mice (*2*).

capacity to differentiate *per se* [2]. Cells from suspension fraction of TNF$^{-/-}$ LTBMC give rise to normal granulocyte-macrophage colonies in semiliquid media; if the suspension fraction cells are injected to lethally irradiated mice, splenic colonies differentiate into granulocytic, erythroid, and megakaryocytic colonies [2]. We can hypothesize that the composition of hemopoietic cells is initially different in WT and TNF$^{-/-}$ LTBMC, but the number of cells with myeloid differentiation markers did not decrease in TNF$^{-/-}$ LTBMC until week 30, which indicates qualitative, but not quantitative differences.

The production of growth factors is usually lower in LTBMC in comparison with the bone marrow. We also know that the induction of expression of granulocyte-macrophage and granulocyte growth factors is markedly reduced in TNF$^{-/-}$ mice [6]. Presumably, the decrease in the concentration of growth factors in the culture prevents differentiation of precursor cells. Accumulation of precursor cells was not associated with disorders in apoptosis and proliferation capacity [1]. The levels of CFU-GM [1], CFCA-7 and CFCA-28 did not differ in cultures of both types. It seems that the absence of TNF determines better survival and slower aging of precursor cell or provides some other advantages.

Hence, new properties of TNF were revealed in LTBMC. The absence of this factor during long-term culturing promotes accumulation of precursor cells of different degree of maturity, while cell capacity to differentiation in culture considerably decreased.

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