# Induced Cell Death and Its Role in Hemopoietic Hypoplasia After Cytostatic Treatment

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The morphological signs of apoptosis in the bone-marrow cells and thymocytes, indices of cellularity in these organs and in peripheral blood and the absolute number of committed bone marrow cells-precursors have been studied on CBA mice injected with Etoposid ($^1/_2$ $LD_{50}$). The results of the study suggest that reduced cellular counts observed in the hemopoietic organs 3-6 h after the cytostatic injection are due to Etoposid-induced apoptosis.

**Key words:** *Etoposid; apoptosis morphology; bone marrow; thymus; peripheral blood*

Recent studies have shown that the cytostatic effect of antitumor drugs relates to apoptosis-programmed cell death [2,6] which is characterized by internucleosomal DNA degradation and disturbed permeability of the plasma membrane. Some nuclear and cytoplasmic modifications associated with apoptosis have been described for cells with and without nucleus [7]. These modifications were chromatin condensation and margination, pycnosis and fragmentation, decay into granules, diminished cell volume, desintegration of a cell into discrete portions (apoptotic bodies) [10-12].

In the present work we studied the morphological signs of apoptosis in mouse thymocytes and bone marrow (BM) cells and changes in cell counts induced by cytostatic treatment.

## MATERIALS AND METHODS

Experiments were performed on 140 male CBA/CaLac mice (2-month-old; 18-20 g). The control group included 16 animals. The mice were housed in accordance with the European Convention on the Care and Use of Laboratory Animals (Strasburg, 1986). Before and during experiments the animals were kept in plastic cages with wooden floor (15 animals per cage) in the vivarium at air temperature of 20-22 °C; 50% humidity or less; outflow/inflow ventilation (8:10); 12-h day/night cycle.

Etoposid (VP-16-213), a podophyllotoxin-like antitumor phytotoxin, inhibits DNA-topoisomerase II, produces cell-cycle arrest in the S and $G_2$ phases, and induces apoptosis [8,9,13]. The latter was demonstrated by electrophoretic DNA analysis in thymocytes, salivary gland cells and in other cells [14,15]. The drug was injected intraperitoneally in a single dose of $^1/_2$ $LD_{50}$ (20 mg/kg) which was determined from graphical probit-analysis for a 30-day observation [1].

Peripheral blood counts of erythrocytes, reticulocytes, plateletes, and leukocytes were determined 0.5, 1, 3, 6, and 12 h, and 2, 3, 4, 5, 7, 14, 21, and 28 days after the cytotoxin injection and averaged in 6-8 animals per each day. The bone marrow cell indices (total count and myelograms) and masses of the spleen and thymus gland were determined after the animals had been killed by cervical vertebral dislocation. Thymocytes and BM cell preparations were stained with azure II-eosin (by Nocht) and investigated for apoptotic bodies and apoptotic cells (pycnosis, fragmentation, nuclear margination, extrusion of apoptotic bodies, etc.). Committed precursor cells of erythro- and granulomonocytopoiesis (CFU-E and CFU-GM) were counted in the tibial bone marrow [4]. Human recombinant erythropoietin (Serva, 0.5 ED/ml) and mouse spleen supernatant (15% medium) activated with phytohemagglu-

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tinin-M (Serva) were used to activate the process. The osmotic resistance of erythrocytes (hemolysis intensity, arb. units) was assessed by their hypertonic resistance [3], intact animals being the control. The results were analyzed with ANOVA statistics using Student's *t* test and Wilcoxon-Mann-Whitney test [5].

## RESULTS

Several hours after injection of Etoposid ($^1/_2$ $LD_{50}$), the signs of cell death were more pronounced in the bone marrow and thymus, than in the spleen (Fig. 1). The number of apoptotic cells and bodies observed in the preparations prepared from the tissue homogenates of these organs in autologous serum (1:1) increased 3 h postinjection of the cytostatic drug (Table 1). In the BM, apoptosis developed predominantly in erythrokaryocytes and lymphocytes and to a lesser extent in granulocytes. The highest count of apoptotic bodies and suicides were observed in the thymus after 6 h, and in the BM after 12 h. The number of apoptotic bodies and other morphological abnormalities in thymocytes and BM cells decreased after 72 h, though remained higher in comparison with the control level.

The total number of BM cells was about 70% of the control value 3 h after injection (Fig. 2, *d*). It was near 40% after 6 h. The most prominent BM hypoplasia was observed on days 3-5. This occurred due to erythrocytopenia that was both early (starting from 3 h) and intense (disappearance of erythronormoblasts started after 12-24 h and lasted till day 7, then partially restored by day 28; Fig. 2, *f*), and due to considerable lymphocytopenia (Fig. 2, *d*).

Erythrocytic osmotic resistance decreased 1 h after injection, and hemolysis intensity remained higher compared with the initial level throughout the entire

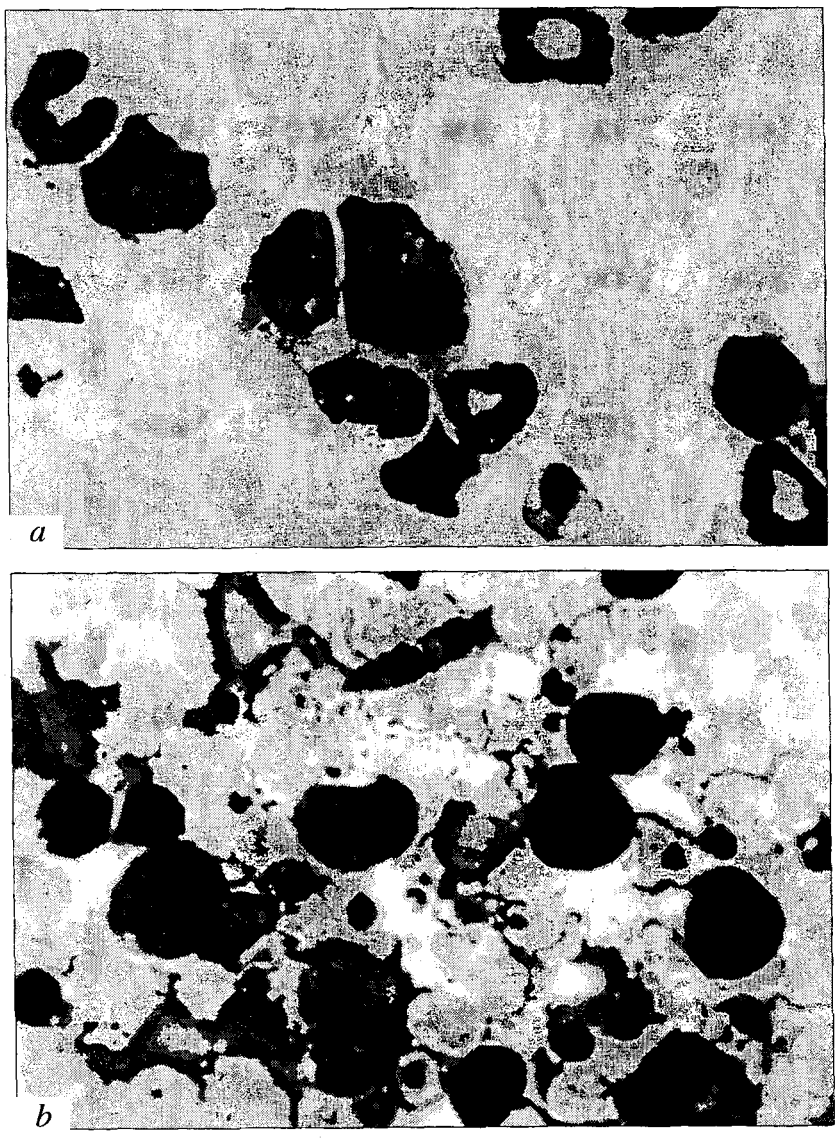


**Fig. 1.** Morphological signs of apoptosis in mouse bone marrow cells at 6 h (*a*) and 3 h (*b*) after injection of Etoposid (20 mg/kg). Apoptotic cells (*a*); apoptotic bodies (*b*). Stained with azure II-eosin, ×1400.

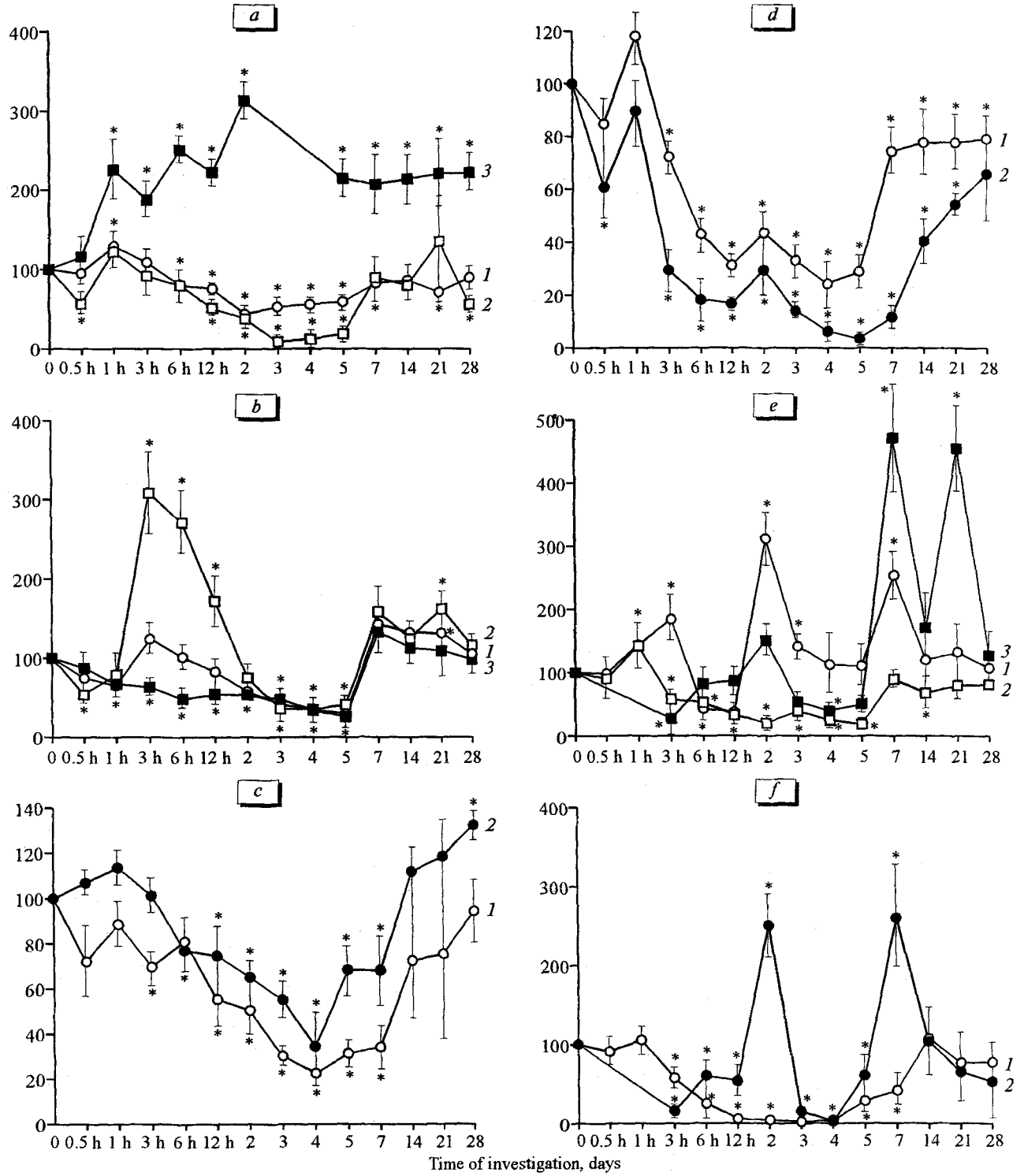


**Fig. 2.** Changes in the parameters of peripheral blood (*a*, *b*), bone marrow (*d-f*), and lymphoid organs (*c*) after injection of Etoposid (20 mg/kg). *a*: erythrocyte (*1*) and reticulocyte (*2*) counts, and hemolysis intensity (*3*); *b*: total numbers of leukocytes (*1*), neutrophiles (*2*), and lymphocytes (*3*); *c*: masses of the thymus (*1*) and spleen (*2*); *d*: total numbers of myelokaryocytes (*1*) and lymphocytes (2); *e*: counts of immature neutrophiles(*1*), mature neutrophiles (*2*), granulocyte-macrophagal colony-growing cells (*3*); *f*: counts of erythrokaryocytes (1), erythrocytic-colony-growing cells (3); ordinate: cell counts in per cent of control (100%). *$p<0.05$ compared with control.

observation period (Fig. 2, *a*). The erythrocyte counts remained decreased from the 6th h to day 21, reaching the minimum on days 2 to 5 (45-60% of the control). Etoposid-induced anemia in mice is probably related to hemolysis, erythrokaryocyte apoptosis, and reduced number of committed BM precursor cells (CFU-E).

**TABLE 1.** Morphological Signs of Apoptosis in CBA-Mouse Thymocytes After Injection of Etoposid (20 mg/kg, $\bar{X}\pm m$)

| Hours | BM | | Thymus | |
|---|---|---|---|---|
| | apoptotic bodies | apoptotic cells | apoptotic bodies | apoptotic cells |
| Control | 0.2±0.1 | 0.1±0.1 | 0.4±0.1 | 1.9±04 |
| 0.5 | 0.3±0.1 | 0.2±0.1 | 0.3±0.1 | 1.8±0.3 |
| 1 | 0.6±0.3 | 0.3±0.1 | 0.2±0.1 | 1.1±0.1 |
| 3 | 3.3±0.8* | 5.1±0.4* | 8.0±1.9* | 4.4±0.5* |
| 6 | 9.0±1.7* | 11.2±0.7* | 34.5±1.6* | 8.8±1.0* |
| 12 | 14.5±1.0* | 12.0±4.0* | 12.0±2.1* | 5.1±1.1* |
| 72 | 1.3±0.4* | 0.5±0.1* | 0.7±0.2 | 2.9±0.6 |

**Note.** *$p<0.05$ compared with control.

Etoposid produced weaker toxic effect on granulocytopoiesis. The depression of immature granulocytes (myeloblasts, promyelo-, myelo-, methamyelocytes) in the BM was short (6-12 h, 40% of the control); depression of mature cells (stab and segmented neutrophils) started at 3 h (60%) and lasted till day 5 (20% of the control). Absolute number of CFU-GM decreased after 3 h and on day 3-5 (Fig. 2, *e*). In peripheral blood leucopenia developed on days 2-5, transient neutropenia on days 3-5, lymphocytopenia and thrombocytopenia from 6 h through day 7 (Fig. 2, *b*).

The lymphoid organs decreased in mass soon (thymus 3 h, spleen 6 h) after Etoposid injection and remained low compared with control as far as till day 7 (Fig. 2, *c*). Thymic hypoplasia was due to thymocyte apoptosis, predominantly of the small lymphocytes. Compared with the thymus, splenic hypoplasia and associated apoptosis were significantly weaker.

Thus, Etoposid damages DNA and induces apoptosis resulting in early hypoplasia of the thymus, BM, and, to a lesser extent, of the spleen and in pancytopenia in the peripheral blood. This cytostatic drug causes profound long-term depression of erythropoiesis, which results in anemia.

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