importance in the understanding of the mechanisms of immunity to smallpox. The resistance and insusceptibility of an animal or vaccinated person to smallpox infection are largely dependent on the state of their cellular immunity. Some demonstrative investigations from this point of view have been carried out by Downie and McCarthy [3], who showed that the presence of smallpox antibodies in high titers in the blood does not completely reflect the state of resistance of the body to smallpox infection.

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INVESTIGATION OF IMMUNOGLOBULIN-POSITIVE CELLS IN MOUSE BONE MARROW

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The number of immunoglobulin- (Ig-) positive lymphocytes and of their precursors in mouse bone marrow was investigated 6 and 36 h after treatment with hydroxyurea. The number of Ig-positive B-cells in bone marrow so treated was increased a little, whereas dividing and nondividing precursors of B-lymphocytes were virtually absent, with the exception of stem cells.

KEY WORDS: bone marrow; B-cells; precursors of B-cells; stem cells.

The problem of fractionation of bone marrow cells in order to isolate early precursors of hematopoiesis and, in particular, stem cells is a problem of continually increasing urgency [4, 8-10]. After treatment of bone marrow with hydroxyurea passage of the cells from the G_1-period into the S-phase is blocked [11, 12] and DNA synthesis is inhibited and the cells die in the S-period [12, 14, 15]. Considerable exhaustion of the pool of proliferating precursors of hematopoiesis has been achieved by means of hydroxyurea [5], so that the subsequent development of these cells takes place almost entirely on account of previously nonproliferating stem cells.

The object of the present investigation was to study the following problems: How does the relative proportion of mature immunoglobulin- (Ig-) positive B-lymphocytes and of colony-forming units (CFU) in bone marrow change under the influence of hydroxyurea; under the same conditions how does the number of proliferating bone marrow cells decrease and, in particular, what is the degree of exhaustion of the pool of early precursers of B-lymphocytes? Finally, what is the quantitative contribution of early (stem cells) and late precursors to the formation of Ig-positive lymphocytes after transplantation of bone marrow into lethally irradiated recipients?

EXPERIMENTAL METHOD

Experiments were carried out on male CBA mice weighing 18-22 g obtained from the Stolbovaya nursery, Academy of Medical Sciences of the USSR.

Hydroxyurea (Serva) was injected intraperitoneally in a dose of 500 mg/kg body weight 4 times at intervals of 5 h between injections [5]. Cell suspensions were prepared from the

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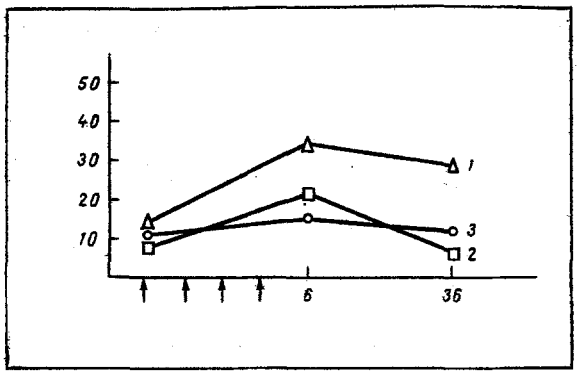

Fig. 1. Number of Ig-positive lymphocytes in bone marrow of mice receiving hydroxyurea. Abscissa, time after last injection of hydroxyurea (in h); ordinate, Ig-positive cells (in % of all nucleated bone marrow cells). 1) Luminescence method; 2) cytotoxic test; 3) autoradiographic method. Arrows indicate injection of hydroxyurea.

bone marrow of the long bones of the hind limbs, the spleen, and the thymus of the animals 6 or 36 h after the last injection of hydroxyurea.

The number of cells carrying immunoglobulins on their surface was determined by three methods. In the immunofluorescence method of determination of the number of cells the indirect Coons' method was used on living cells fixed to slides by means of a formvar film [1]. Rabbit serum against mouse globulins and fluorescent donkey serum against rabbit globulin (N. F. Gamaleya Institute of Epidemiology and Microbiology) were absorbed twice with an acetone powder of mouse liver. The number of Ig-positive cells also was determined by the cytotoxic test, using rabbit antiserum against mouse globulins and complement. Finally, the globulin fraction isolated from rabbit antiserum against mouse globulins (with sodium sulfate at 40% saturation) was labeled with ^{125}I-chloramine T by the method of Greenwood et al. [7]. The specific activity was 4 μCi/μg protein. To 0.2 ml of the suspension ($5 \cdot 10^6$-$10 \cdot 10^6$ cells) 0.05 ml of labeled protein (5 μg) was added and the mixture was incubated at 4°C for 30 min in medium with 0.1% sodium azide (Serva) and 10% embryonic calf serum (Serva). The cells were washed 3 times, after which films were prepared, fixed with ethanol, and coated with type M (State Photographic Chemical Research Institute Project) photographic emulsion. The number of DNA-synthesizing cells was determined by labeling *in vitro* with ^{3}H-thymidine (5 μCi/ml) for 1 h.

To determine the degree of exhaustion of the pool of precursors of B-lymphocytes experiments were carried out in which bone marrow cells, treated by various methods, were transplanted into lethally irradiated (950 rad) syngeneic recipients. Cells carrying receptors against the hapten — the trinitrophenyl (TNP) group — were detected by the rosette-formation test with sheep's red blood cells prepared by the method of Rittenberg and Pratt [13]. Some of the recipients' spleens were embedded in paraffin wax and sections were stained with hematoxylin-eosin and methyl green-pyronine.

To determine the number of CFU in the bone marrow $4 \cdot 10^4$ cells were injected into lethally irradiated recipients. The number of hematopoietic colonies in their spleens was counted 8 days later. In some experiments the donors' cells were treated with antimarrow antiserum (antistem-cell antiserum), prepared and absorbed as described by Golub [6], and with complement. Preliminary tests showed that this serum reduced the number of CFU in the bone marrow by 24 times. In a separate experiment rabbit antimarrow antimouse serum was absorbed successively with mouse red blood cells, liver homogenate, and acetone liver powder and used as anti-T-serum for immunoluminescence analysis. In the cytotoxic test it killed 30% of spleen cells and 85-95% of thymus cells.

EXPERIMENTAL RESULTS

A sharp decrease in the absolute number of cells (by 80% in the bone marrow) was observed in the bone marrow and thymus 6 h after the last injection of hydroxyurea. The number of cells in the spleen and lymph nodes fell by 20-30%. The proportion of blast cells was

TABLE 1. Results of Investigation of Spleens of Lethally Irradiated Mice 8 Days after Irradiation and Transplantation of Bone Marrow Cells ($1.5 \cdot 10^5$)

Source of bone marrow	Number of TNP-RFC			
	per 10^6 cells	per spleen	inhibition by TNP-BSA, %	inhibition by anti-Ig-serum, %
No bone marrow cells injected into recipients	2 500	2 800	Not inhibited	—
Intact donors	10 000	40 000	75	60
Donors receiving hydroxyurea	3 200	42 000	84	78
Bone marrow cells of intact donor, treated with antistem-cell serum and complement	3 700	5 500	60	60

sharply reduced and the number of mature cells increased. The fraction of DNA-synthesizing cells suffered a 40-fold reduction (from 16.2 to 0.4%).

After 36 h the number of nucleated cells in the bone marrow still continued to decline whereas the number of blast cells was significantly increased. These blast cells exhibited morphological homogeneity, by contrast with their heterogeneity in intact mice. The number of DNA-synthesizing cells increased up to 9.8%.

Most of the cells binding ^{125}I-labeled anti-Ig-antibodies in the bone marrow of the normal mouse are small lymphocytes (over 70%). In the early stages after injection of hydroxyurea the proportion of Ig-positive cells increased (Fig. 1) and the number of labeled blast cells was reduced by two-thirds.

Immunoluminescence analysis of the bone marrow by means of rabbit anti-T-serum revealed small lymphocytes, on a small prominent area of the surface of which marrow antigens were concentrated, in the intact mice and, in particular, in mice receiving hydroxyurea. These cells had no immunoglobulin molecules on their surface. They were probably prothymocytes [3].

In the experiments in which bone marrow obtained 6 h after injection of hydroxyurea into the donors was transplanted into lethally irradiated recipients the number of CFU was not increased compared with the number in the bone marrow of intact animals. Treatment of both types of cells with antibrain-stem serum abolished colony formation; no discrete or diffuse cell proliferation could be found morphologically in the recipients' spleens.

After treatment of the donors' cells with antistem-cell serum, in order to determine the quantitative contribution of the earliest precursors (sensitive to antistem-cell serum) to B-lymphocytopoiesis and clonogenesis, and to assess the contribution of other precursors and of mature B-lymphocytes, a small number of rosette-forming cells specific for TNP (TNP-RFC; Table 1) was found in the recipients' spleens. The number of TNP-RFC was one order of magnitude higher after transplantation of intact bone marrow cells or bone marrow cells from donors receiving hydroxyurea. After transplantation of bone marrow, for instance, about 90% of specific antigen-binding B-lymphocytes thus developed from precursors sensitive to anti-marrow serum.

Injection of hydroxyurea into mice for 21 h caused marked exhaustion of the pool of proliferating cells and a decrease in the absolute number of nucleated cells in the bone marrow. A sharp decrease in the number of cells also was observed in the thymus. The increase in the number of Ig-positive cells in the bone marrow did not correspond to the fivefold decrease in the number of nucleated cells. This can be explained by the existence of two parallel processes: the outflow of mature B-lymphocytes from the bone marrow and death of their proliferating precursors.

Judging from the sudden exhaustion of the proliferative pool in the bone marrow of the mice receiving hydroxurea, the relatively late precursors of B-lymphocytes must have been virtually absent. This was confirmed by the results of experiments in which suspensions of bone marrow cells treated with antistem-cell serum were transplanted, which showed that after four injections of hydroxyurea virtually no precursors of hematopoiesis remained in the do-

nors' bone marrow (except cells sensitive to antimarrow serum, i.e., CFU), capable of proliferating and developing in the spleen of lethally irradiated recipients. The contribution of these early precursors (stem cell?) to B-clonogenesis was one order of magnitude higher than the contribution of the remaining precursors and of relatively mature B-cells.

These experiments showed that the number of CFU in the bone marrow of mice receiving hydroxyurea is not increased, possibly because the hydroxurea caused death of stem cells which had started to undergo proliferation. Evidence of the stimulation of their proliferation is given by the 30-40-fold increase in the number of CFU observed in [2] after 36 h.

The number of CFU in the bone marrow of mice receiving hydroxyurea in accordance with the scheme described above is thus not greater, when expressed in proportion to the number of nucleated cells, than in the bone marrow of intact mice. The number of Ig-positive B-cells was increased a little, whereas dividing and nondividing precursors of B-lymphocytes were virtually absent. Such bone marrow is a convenient model for the successive (stage by stage) study of B-lymphocytopoiesis and B-clonogenesis, beginning at a definite starting point.

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