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MAKING ALLOGENEIC BONE MARROW TRANSPLANTATION MORE EFFECTIVE

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During allogeneic bone marrow transplantation, there is the risk of development of immunologic conflicts: recipient versus graft and graft versus host (DVHR) reactions. If, however, a mixture of genetically different cells from two or more donors is transplanted, immunologic conflict arises between the donors' cells: a graft versus graft reaction [2, 7, 9]. This gives rise to serious adverse effects, for inhibition or complete blocking of proliferation of the stem cells of one donor under the influence of nonsyngeneic lymphocytes can sharply reduce the therapeutic effect of a mixed graft [5].

In clinical practice in the treatment of depressions of hematopoiesis, it is often necessary to use large doses of bone marrow from several donors, and for that reason the development of an effective method of conservation of allogeneic bone marrow, enabling the activity of the immunocompetent cells of the graft to be reduced, would be promising.

The investigation described below was devoted to a search for ways of making allogeneic bone marrow grafting more effective by measures aimed at particular populations of lymphocytes (T lymphocytes) of the graft.

EXPERIMENTAL METHOD

The immunologic activity of lymphocytes located in bone marrow and lymph nodes was determined by methods of estimating inactivation of nonsyngeneic stem cells and abolition of endogenous colony formation.

Mice of strains CBA and C57BL/6 were used as donors, (CBA × C57BL/6)F_1 hybrids as recipients. A cell suspension was prepared in TsOLIPK-3* conserving solution. The recipient mice were irradiated in a lethal dose (880 R). Lymph nodes or bone marrow cells from CBA mice were

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TABLE 1. Measurement of Exogenous Colony Formation (macroscopic counting) after Injection of Frozen Bone Marrow from Genetically Different Donors into Lethally Irradiated Recipient

Group of animals	Number of cells injected		Mean number of colonies (M±m)	Inactivation index, %
	CBA	C57BL/6		
1	$0,5 \cdot 10^6$	$0,5 \cdot 10^6$	7,4±0,1	0
2	$1,0 \cdot 10^6$	$0,5 \cdot 10^6$	11,6±0,09	0
3	$2,0 \cdot 10^6$	$0,5 \cdot 10^6$	18,9±0,05	0
4	$4,0 \cdot 10^6$	$0,5 \cdot 10^6$	26,3±0,04	0
5	—	$0,5 \cdot 10^6$	0,9±0,04	—
6	$0,5 \cdot 10^6$	—	4,0±0,07	—
7	$1,0 \cdot 10^6$	—	5,6±0,1	—
8	$2,0 \cdot 10^6$	—	10,0±0,1	—
9	$4,0 \cdot 10^6$	—	16,1±0,3	—
10	Irradiation control		0,3±0,01	—

TABLE 2. Changes in Inactivation of Endogenous CFU after Transplantation of Glycerol-Tested Cells from Lymph Nodes of CBA Mice into Sublethally Irradiated (600 R) (CBA × C57BL/6)F_1 Recipients

Number of lymph node cells from CBA mice injected	Intact cells		Cells treated with 15% glycerol				Cells treated with 7% glycerol		Cells treated with 5% glycerol	
			without rinsing		with rinsing					
	number of colonies in spleen, (M ± m)	inactivation index, %	number of colonies in spleen, (M ± m)	inactivation index, %	number of colonies in spleen, (M ± m)	inactivation index, %	number of colonies in spleen, (M ± m)	inactivation index, %	number of colonies in spleen, (M ± m)	inactivation index, %
$0,5 \cdot 10^6$	5,0±0,5	56,2	7,4±0,5	26,8	11,8±0,4	4,6	8,0±0,3	10,1	9,3±0,3	41,1
$1,0 \cdot 10^6$	0,23±0,1	97,7	12,7±0,3	0	9,8±0,2	19,7	7,4±0,2	16,9	4,3±0,3	73,6
$2,0 \cdot 10^6$	0,04±0	100	13,1±0,3	0	6,6±0,4	46,0	3,7±0,2	58,4	0,6±0,05	96,3
$4,0 \cdot 10^6$	—	—	13,5±0,3	0	1,1±0,09	91,0	0,6±0,03	93,3	0,2±0,01	98,7
—	11,4±1,9	—	10,1±0,2	—	12,2±0,08	—	8,9±0,2	—	15,8±0,3	—

treated with 5, 7, and 15% glycerol. In the series of experiments in which freezing with 15% glycerol was used, the conditions were the mildest possible for stem cells: 1°C per minute from the original temperature (18-20°C) to the beginning of crystallization (−9 to −10°C), followed by 10°C/min to −196°C. On the 9th day after transplantation of the cells, the number of colonies in the recipient's spleen was counted.

In each experiment, morphologically preserved cells were counted by supravital staining with eosin and the number of nucleated cells was counted in a Goryaev's chamber. On transplantation of the cell suspension into recipient animals, the dose was based on the number of "living" cells.

To study the effect of different glycerol concentrations on immunologic activity of T and B lymphocytes in the donors' peripheral blood, the method of allogeneic and heterogeneic rosette formation was used.

EXPERIMENTAL RESULTS

Effect of Conservation Factors on Homotransplantation Activity of Lymphocytes. Previous investigations in the writers' laboratory showed that the homotransplantation activity of lymphocytes contained in lymph nodes, spleen, and bone marrow falls sharply as a result of freezing (15% glycerol was used as the cryoprotective agent). These experiments showed that to achieve an effect of inactivation of nonsyngeneic C67BL/6 stem cells (targets) by frozen CBA lymphocytes (killers), 8 times more of the latter than of native (not frozen) cells are needed [1, 4].

The next series of experiments were conducted on bone marrow; not only the killer cells, but also target cells were frozen in 15% glycerol, after which cells of the mixed frozen graft were transplanted into lethally irradiated recipient mice. Under these conditions, the frozen killer cells had no inactivating effect on the target cells, not only if present in equal numbers (1:1), but also when the number of killer cells was increased by 2, 4, and 8 times. Conversely, the number of colonies in the lethally irradiated recipient increased and was greater than the total number of colonies obtained from injection of cells from each donor separately (Table 1). These results indicate that when allogeneic bone marrow from several donors, conserved with 15% glycerol, is transplanted into a recipient animal, it may not only reduce inactivation of the stem cells by nonsyngeneic lymphocytes, but may also have the effect of potentiating exogenous colony formation.

Consequently, when frozen bone marrow from genetically different donors is transplanted into a recipient, not only is the danger of incompatibility of lymphocytes and stem cells reduced, but its therapeutic effect is enhanced. The possibility cannot be ruled out that transplantation of bone marrow conserved by freezing may in some cases be clinically more effective than transplantation of fresh bone marrow. It is also evident that when bone marrow conserved by freezing is transplanted, the view that bone marrow from two or more genetically different donors cannot be transplanted requires revision.

Definite conclusions on the pathogenesis of the phenomena described above can also be drawn from the results of this investigation: conservation factors act mainly on a particular lymphocyte population, namely, T lymphocytes. This is confirmed by the fact that convincing evidence of a reduction of the "killer effect" was obtained in the experiments, and we know that this effect is characteristic of T lymphocytes, whereas B lymphocytes have no killer properties [3].

To study which of the conservation factors — the freezing process itself or the cryoprotector, glycerol, is responsible for reducing the homotransplantation activity of lymphocytes, a series of experiments was carried out in which cells from lymph nodes were treated with different concentrations of glycerol (without freezing). The action of 5, 7, and 15% glycerol on lymphocytes (with and without rinsing) was studied, and later, in experiments *in vivo*, the activity of these lymphocytes was assessed during their interaction with nonsyngeneic stem cells. Altogether, 1200 F_1 hybrid mice and 150 CBA mice were used in the experiments.

The experiments showed that treatment of lymphocytes with 15% glycerol leads to abolition of the inactivating effect against nonsyngeneic stem cells (Table 2). Washing the cells to remove glycerol did not completely restore the immunological activity of the lymphocytes. It remained low (19.7 and 46.0% instead of 97.7 and 100%). A similar effect on the lymphocytes was given by 7% glycerol. When this concentration was used, the inactivating activity decreased to 58.4-16.9% depending on the dose of cells. Only with a fourfold increase in the dose of cells treated with 7% glycerol was a marked (93.3%) inactivating effect observed. Treatment of the cells with 5% glycerol had no such effect. Even the minimal dose (0.5 ± 10^6) gave high (41.1%) inactivation of endogenous colony-forming units (CFU; see Table 2).

The experiments thus showed that 7 and 15% glycerol reduce the activity of lymphocytes against nonsyngeneic CFU. The establishment of this fact is very important because it means that simple treatment of hematopoietic tissues with glycerol alone (without freezing) is sufficient to weaken or abolish the inactivating effect of lymphocytes against nonsyngeneic stem cells. The use of this convenient method under clinical conditions to make transplantation of allogeneic bone marrow more effective is very promising. The method is also very convenient and effective when it is necessary to transplant bone marrow from several genetically different donors into a recipient.

Weakening of Secondary Sickness in Lethally Irradiated Animals after Transplantation of Conserved Hematopoietic Tissue. The effect of conservation with 15% glycerol on the reduction in activity of immunocompetent cells responsible for development of the GVHR was studied.

Cells from lymph nodes, spleen, and bone marrow of CBA mice were conserved by freezing by the method described above. The conserved cells were injected into (CBA × C57BL/6)F_1 hybrid recipients 20 h after lethal irradiation. The GVHR was assessed by the survival of recipients for 30 days after transplantation, and changes in body weight and weight of the spleen (the index of enlargement of the spleen was calculated by means of an equation). Spleens of animals which died were studied histologically. Altogether, 1200 F_1 hybrid mice and 150 CBA mice were used in the experiments. The experiments showed (Fig. 1) that after transplantation of frozen lymph node cells, the symptoms of secondary sickness in the animals were alleviated and their

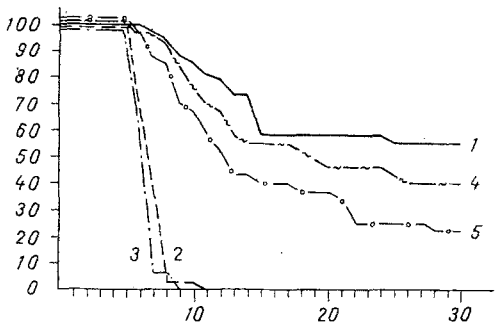

Fig. 1

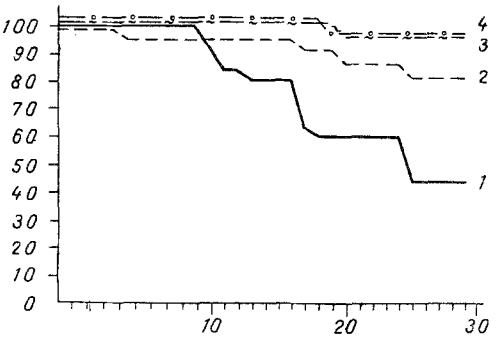

Fig. 2

Fig. 1. Survival rate of lethally irradiated (CBA × C57BL/6)F_1 hybrids after transplantation of lymph node cells from CBA mice: 1) irradiation control; 2) intact lymph node cells (1×10^7); 3) intact lymph node cells (2×10^7); 4) proliferated lymph node cells (1×10^7); 5) proliferated lymph node cells (2×10^7). Here and in Fig. 2: abscissa, days after transplantation; ordinate, % of surviving animals.

Fig. 2. Survival of lethally irradiated (CBA × C57BL/6)F_1 hybrid mice after transplantation of bone marrow cells from CBA mice. 1) Irradiation control; 2) intact bone marrow cells (1×10^7); 3) proliferated bone marrow cells (1×10^7); 4) frozen bone marrow cells (2×10^7).

TABLE 3. Effect of Glycerol in Different Concentrations on Rosette-Forming Ability of Donors' Peripheral Blood Lymphocytes (M ± m)

Suspension of peripheral blood lymphocytes	Proportion of lymphocytes, %	
	T	B
Without glycerol	55,8±3,2	25,5±1,9
With 15% glycerol		
without rinsing	20,2±3,0	16,2±0,9
with rinsing	19,3±2,7	15,2±2,2
With 7% glycerol	36,8±4,6	22,2±2,5
With 5% glycerol	24,8±1,4	18,7±1,9
With 3.3% glycerol	36,1±3,6	22,1±2,1

survival rate was 40-50% by the 15th day and 20-40% by the 30th day (in the control group, mortality of the animals was 100% by the 9th-10th day). Similar results were obtained after freezing spleen cells.

When frozen allogeneic bone marrow was transplanted into the animals (Fig. 2), the symptoms of secondary sickness were alleviated and the survival rate of the animals was high (96% by the 30th day of observation). On histological investigation of the spleens of recipients of frozen lymph nodes, spleen, or bone marrow cells, no morphological signs of secondary sickness was found: the structure of the organ was preserved and plasmatization of the cells was not observed.

The experiments thus showed that conservation of lymphoid and hematopoietic tissues by freezing and the protection by 15% glycerol sharply reduces the possibility of development of secondary sickness, and in turn, this ensures better survival and function of the allografts.

Effect of Glycerol on Ability of T and B Lymphocytes to Participate in the Heterogeneic Rosette Formation Reaction. The effect of different concentrations of glycerol on immunologic activity of T and B lymphocytes from the donors' peripheral blood was studied. To determine the level of immunologic activity of the lymphocytes, methods of E-rosette formation [8] and EAC-rosette formation [6] were used with modifications. The results of these experiments are given in Table 3.

Experiments with the donors' peripheral blood confirmed the previous observations in experiments *in vivo*, showing that treatment with glycerol affects the immunologic activity of the lymphocytes through its selective action on the T lymphocyte population.

The new property of glycerol, revealed by these experiments, of reducing the homotransplantation activity of T lymphocytes thus suggests that conserved bone marrow has advantages over freshly prepared marrow because it weakens the GVHR and possesses higher proliferative activity. In addition, if conserved bone marrow is transplanted from several donors, there is no risk of inactivation of the stem cells by nonsyngeneic lymphocytes and exogenous colony formation in the recipient is enhanced.

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ANALYSIS OF BLOOD SERUM AFTER INJURY TO THE SUBMANDIBULAR SALIVARY GLAND IN RATS

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The writers have shown that sialotomy of the submandibular salivary gland (SMSG) activates proliferation from a distance in the corneal epithelium [6]. If the phenomenon were specific for SMSG, it could be postulated that it is attributable to high-molecular-weight polypeptide growth factors synthesized by the gland: epidermal (mol. wt. 74,000), endothelial (mol. wt. 80,000-86,000) [2], and mesodermal [14]. The present writers also found hyperplasia of the corneal epithelium after trauma to the liver [6]. It is worth mentioning that other nonspecific mitogens also exist.

The object of the present investigation was to compare changes in the protein, glycoprotein, enzymic, and antigenic composition of the blood in the course of time after partial resection of SMSG in order to detect any possible regeneration mitogens.

EXPERIMENTAL METHOD

Experiments were carried out on 129 Wistar rats weighing 140-160 g, of both sexes. Trauma to SMSG was produced by the method described previously [6]. Serum protein fractions were studied by electrophoresis in agarose gel. The seromucoid content was determined by the modification in [5]. Proteins of the traumatized and contralateral SMSG were detected in the blood by double diffusion in agar as in [4]. A saline extract of SMSG tissues obtained from 140 intact rats was used as the antigen. Antisera were obtained by immunizing six mature rabbits 9 times. The antiserum was exhausted of antibodies against blood proteins by fractional absorption with

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