IMMUNOLOGY AND MICROBIOLOGY

Effects of Immunization with Group A Streptococcal Antigens on the Transplantability of Mouse Bone Marrow Stromal Stem Cells, Counts of Stromal Precursor Cells, and Their Osteogenic Characteristics

Yu. F. Gorskaya, T. A. Danilova, O. V. Lebedinskaya, V. G. Lunin*, V. I. Grabko*, N. E. Sharapova*, and V. G. Nesterenko**

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 151, No. 5, pp. 544-548, May, 2011
Original article submitted April 13, 2010

Immunization of CBA mice with killed group A streptococcus (type 5) vaccine changed the counts of stromal precursor cells (CFC-F) in bone marrow transplants at different donor-recipient combinations (normal, N, or immune, I). CFC-F counts in bone marrow transplants from normal mice transplanted to immunized animals decreased 4-6-fold depending on the transplant age in comparison with similar transplants in normal recipients. The percentage of CFC-F colonies with alkaline phosphatase (osteogenesis marker) activity decreased more than 2-fold. Similarly, the count of CFC-F in the transplants was 2-fold lower during delayed (7 months) period after bone marrow transplantation from immunized donors (8-12 days after the end of immunization) to intact recipients, while 2 months after transplantation it was 3-fold lower. The mean optical density of the bone capsule in preparations stained for glycogen and alkaline phosphatase was 1.5-3 times lower in the N→I and I→N experiments in comparison with the control (N→N). On the other hand, CFC-F count in the femoral bone marrow of immunized animals was significantly (3.5-2.5 times) higher during the period from 8 days to 8 months after the end of immunization compared to CFC-F count in the femoral bone marrow of intact mice. These results attest to a significant prolonged effect of streptococcal antigens on the bone marrow stromal tissue. These data also indicate that not all CFC-F, the counts of which increased in response to antigens, are responsible for transplantability of the stromal tissue in heterotopic transplantation. Immunization by streptococcal antigens seemed to suppress transplantability and osteogenic activity of stromal stem cells. The efficiency of CFC-F cloning in mouse bone marrow cultures increased significantly (2-3-fold) in the presence of sera from immune mice. The levels of TNF-α and IFN-γ were low in this serum (2.7 and 6 times lower, respectively) in comparison with normal serum. Presumably, the effects of streptococcal antigens on stromal tissue were mediated through serum cytokines.

Key Words: *bone marrow stromal cells; immune response; streptococcal antigens*

Laboratory of Immunity Regulation, *Laboratory of Bioactive Nanostructures, N. F. Gamaleya Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences, Moscow; **Department of Histology, Perm State Medical Academy, Russia. ***Address for correspondence:*** Uliya.Gorskaya@nearmedic.ru. Yu. F. Gorskaya

The bone tissue is continuously restructured throughout the entire human life. The organism faces a variety of infections throughout life, and the bone tissue can be partially reorganized by stromal stem cells (SSC) under conditions of an unfolding infectious process. The problem is how an infectious process and the autoimmune state often developing after it influence so important cell category as stromal tissue and what are the aftereffects (immediate and delayed) of these situations. We tried to answer this question on a model of heterotopic transplantation of the bone marrow in animals immunized with streptococcal antigens. This infection is, on the one hand, highly prevalent, and on the other, it often provokes the development of autoimmunity. Transplantation of bone marrow fragments under the renal capsule leads to the development of a bone marrow organ populated by recipient hemopoietic cells from donor SSC at the site of transplantation within 2-3 weeks [9,11]. The status of this organ indicates the efficiency of its formation under certain conditions, in our case after donor or recipient immunization with streptococcal antigens. It is known that the immune process is associated with changes in the spectrum and levels of cytokines in the blood. At least some of them can modulate the bone tissue [15], the growth and proliferation of bone marrow stromal precursor cells (CFC-F) and their descendants (fibroblasts from strains cultured throughout several passages) *in vitro* [2]. We tried to solve several problems: to trace changes in CFC-F counts in bone marrow transplants in various combinations of normal and immunized (with streptococcal antigens) donors and recipients during different periods after immunization of animals and to evaluate (1) the effects of immunization with streptococcal antigens on CFC-F count in the bone marrow of immune animals; (2) the histochemical characteristics of heterotopic bone marrow transplants of immunized mice in different donor-recipient combinations; (3) possible effects of immune *vs.* normal mouse serum on cultured bone marrow CFC-F growth; and (4) serum levels of cytokines (TNF-α, IFN-γ, IL-2, -4, -5, -10, -12, and granulocytic macrophage CSF (GM-CSF) in mice immunized with streptococcal antigens in comparison with normal mice.

MATERIALS AND METHODS

Experiments were carried out on 2-3-month-old CBA mice and 4-5-month-old guinea pigs from Kryukovo Breeding Center. Mice were immunized with killed streptococcus A (type 5) vaccine (kind gift from N. A. Borodiyuk, Doct. Biol. Sc., Laboratory of Immunity Regulation, N. F. Gamaleya Institute). The method of vaccine preparation was described previously [3]. The mice were immunized over 3 weeks with the vaccine in ascending doses injected intraperitoneally 3 days running: 1 billion bacterial cells per injection on week 1, 2 billions on week 2, and 3 billions on week 3. After immunization by this protocol, the animals produce antibodies to not only streptococcal antigens, but also autoantibodies to body tissue antigens, the maximum antibody production was observed on days 7-14 after the last injection of the antigens [1,7]. Autoantibodies to heart tissue antigens in the sera of immunized mice were evaluated by indirect immunofluorescent method on bovine heart sections. Serum to mouse immunoglobulins, labeled with FITC (N. F. Gamaleya Institute) was used. The methods for staining of sections and interpretation of the results have been described previously [7]. For heterotopic transplantation, a half of mouse femoral bone marrow was injected under the renal capsule of animals [11] in the following donor–recipient combinations: normal–immune (N→I), immune–normal (I→N), and normal–normal (N→N). The counts of CFC-F in the femoral bone marrow and in bone marrow transplants were evaluated by the number of stromal fibroblast colonies, formed by these cells after explantation of the respective cell suspensions in monolayer cultures. Mouse and guinea pig bone marrow cell suspensions and mouse heterotopic transplants were prepared as described previously [3,12] and explanted ($5\text{-}20\times10^5$) into 25-cm^2 flasks in 5 ml α-MEM (Sigma) with 5% FCS (PanEco). The medium with nonadherent cells was discarded after 2 hours, the cultures were washed 2 times in α-MEM, and complete culture medium containing α-MEM (80%), FCS (20%), and antibiotics (penicillin and streptomycin in concentrations of 100 μg/ml) was added. Bone marrow cells (10^7) from guinea pigs exposed in a dose of 60 Gy (Co 60, 10 Gy/min) were added to all cultures as a feeder. The cultures were incubated for 12 days in a CO_2 incubator at 37°C, fixed in ethanol, stained with azur-eosin, and the colonies consisting of at least 50 fibroblasts were counted. Cloning efficiency (CFE-F), *i.e.* the number of stromal fibroblast colonies formed by 10^5 explanted cells, was evaluated by the number of colonies. In order to evaluate morphohistochemical characteristics of the transplants, histological preparations were stained with hematoxylin and eosin, azur-eosin, by van Gieson method, by Brachet method for RNA with RNase control, PAS reaction was carried out after Shabadash with amylase control (for glycogen and neutral glycosaminoglycanes), and alkaline phosphatase activity was evaluated by Gomori's method [8]. Blood was collected in mice on day 8 after immunization. Sera of normal and immune mice were obtained by the standard method, divided into aliquots, and frozen at -20°C. The serum was defrosted and filtered through HA millipore filter directly before use. Normal and

immune sera filtered through HA were added (5-20 μl/ml culture medium) into part of intact bone marrow cultures 10 min after cell explantation. Serum levels of cytokines (TNF-α, IFN-γ, IL-2, -4, -5, -10, -12, and GM-CSF) were measured on a BioPlex device using a kit of appropriate reagents for mice (BioRad). The results were processed using BioPlex Manager Software.

RESULTS

Immunization of CBA mice with killed streptococcus vaccine resulted in the production of autoantibodies detected in the serum by indirect immunofluorescence on bovine heart sections. Antibodies reacting with antigens of sarcolemma and subsarcolemma of myocardial fibril were detected. It was previously shown that these antibodies were directed to organ-specific myocardial antigens, common for animals of different species (rabbit, mouse, bull, *etc.*), in other words, autoantibodies [1,7]. Hence, immunization of animals by the above protocol led to production of not only antibodies to streptococcal antigens, but also of autoantibodies.

The counts of CFC-F in the bone marrow transplants from normal mice injected to immune animals 8 days after their immunization were 4-6-fold lower (depending on the transplant age) in comparison with similar transplants injected to normal recipients (Table 1). The percentage of CFC-F colonies with alkaline phosphatase activity (osteogenesis marker) was more than 2-fold lower. The levels of CFC-F in 1.5-3-month-old transplants of the bone marrow from immunized animals, injected to normal mice, were virtually the same as in bone marrow transplants from normal mice transplanted to normal mice, while in 7-month transplants the content of these colonies was 2-fold lower than in the N→N transplants. The levels of CFC-F in old (7 months) transplants (I→N) were also 2-fold lower and after 2 months 3-fold lower after transplantation of the bone marrow from immune donors 8-12 days after the end of immunization to intact recipients (Table 2). The bone marrow of immunized donors can form in normal recipients a transplant with CFC-F content close to the normal level only 6 months after immunization, and only 6 months after immunization of recipients the transplanted normal bone marrow can provide the level of CFC-F close to the norm in the transplants.

Interestingly, the weight and size of bone capsules of bone marrow transplants collected 2 months after donor immunization was 3-fold lower in 6.5-month-old transplants in comparison with the transplants from intact donors [5]. Comparative analysis of histochemical characteristics of the bone capsule in animals of the control (N→N) and experimental (N→I and I→N) groups showed a lower mean optical density of the bone capsule in experimental groups in the preparations stained for glycogen and in evaluation of alkaline phosphatase activity (1.5-3 times). Hence, the activity of alkaline phosphatase, glycogen and glycosaminoglycane levels were lower in experimental groups, indicating a reduction of osteogenic activity of stromal cells in heterotopic transplants [8]. These data suggest that the transplantability (capacity to construct a new full-value bone marrow organ and maintain the normal level of CFC-F in it) of stromal tissue is disordered in immunized organism. This can be a mechanism of osteoporosis associated with infections and autoimmune diseases. These changes can also lead to disorders in the microenvironmental functions of stromal tissue and disorders in hemo- and lymphopoiesis associated with them. It has been shown that the capacity of the stromal sublayer to support the growth of $CD34^+$ cells

TABLE 1. CFE-F in Bone Marrow Transplants from Normal Mice and Mice Immunized with Killed Group A Streptococcus Vaccine 8 Days after Immunization (*M*±*m*)

Transplant type	Transplant age, months	Number of nuclear cells per transplant, $\times 10^6$	CFE-F, $\times 10^5$	Content of CFC-F in a transplant
N→N	1.5	1.2±0.2	6.7±0.7	80±8
N→I		0.9±0.2	1.9±0.2	17±2
И→H		1.8±0.4	3.8±0.3	68±5
N→N	2.5-3	3.6±0.2	1.3±0.2	47±7
N→I		3.6±0.6	0.3±0.1	11±3
I→N		5.2±0.6	0.8±0.2	41±10
N→N	7-7.5	6.2±1.1	0.5±0.1	33±6
N→I		3.9±0.7	0.1±0.0	5±1
I→N		3.9±0.8	0.4±0.1	15±3

TABLE 2. CFE-F in Bone Marrow Transplants from Normal Mice and Mice Immunized with Killed Group A Streptococcus Vaccine during Delayed Periods after Immunization (*M*±*m*)

Transplant type	Month after the end of immunization	Month of transplant fixation	Number of nuclear cells per transplant, $\times10^6$	CFE-E, $\times10^5$	Content of CFC-F in transplant
N→N	2	1	2.9±0.3	5.3±0.4	145±20
I→N			3.4±0.4	1.8±0.3	55±5
N→N	6	2	3.8±1.1	1.8±0.2	70±12
N→I			4.4±0.9	2.0±0.2	92±15
I→N			4.4±1.0	1.6±0.2	70±15

was disturbed in long-term cultures of bone marrow cells from patients with rheumatoid arthritis [13]. Interestingly, the level of CFC-F in the femoral bone marrow, used for transplantation from 8 days to 8 months after immunization of animals, was also significantly higher (3.5-2.5 times) than CFC-F content in the femoral bone marrow of normal mice (Table 3).

However, this excess was inessential for the size of the transplant forming from this bone marrow or for CFE-F and CFC-F content in it. These data seem to indicate that not all CFC-F whose count increases after antigen injection are responsible for stromal tissue transplantability in its heterotopic transplantation. Hence, the fact that the bone marrow intended for transplantation is CFC-F-rich does not yet predict a more effective formation of the transplant. These data are presumably important for the choice of methods for CFC-F pool enrichment for subsequent transplantation. Importantly that CFC-F population is rather heterogeneous and contains SSC (mesenchymal stem cells) and more mature stromal precursor cells of various differentiation directions [11,14]. Presumably, the count of stromal precursors in the bone marrow after immunization increases at the expense of better differentiated cells. The reduction of SSC transplantability, in turn, can result from switch-over of part of them from "osteogenic" to another differentiation direction, needed for immune response maintenance. Addition of sera from normal and immunized animals to bone marrow cell cultures led to an increase of CFE-F 2-2.5 times in the presence of immune *vs.* normal serum (Table 4). Addition of normal and immune serum sharply increased the CFE-F in comparison with the cultures with FCS alone, the CFE-F in the cultures with immune serum remaining 2-2.5 times higher *vs.* that in cultures with normal serum. These results are in line with our previous data indicating that addition of adult animal serum to FCS-containing cultures can result in CFE-F increase due to arrest of !the CFC-F growth inhibitory effects of macrophages present in the culture [6]. The level of TNF-α was reduced significantly (2.7 times) in immune *vs.* normal serum, as well as IFN-γ level. The levels of IL-2, -4, -5, -10, and -12 were about the same in both sera, while GM-CSF was not found [4]. Our previous data indicating that an increase of TNF-α and IL-1 concentrations in culture media led to inhibition of CFC-F growth and even its complete arrest [2] suggested that reduction of TNF-α concentration in the serum from immunized animals led to an increase of CFE-F by abolishing the inhibitory effect of this cytokine on stromal tissue. However, the immune serum presumably contains some other factors stimulating proliferation of CFC-F, which are still to be identified.

Hence, the results indicate a significant prolonged effect of streptococcal antigens on bone marrow stromal tissue, specifically, on its osteogenic capacity. It seems that immunization stimulates the total count of

TABLE 3. CFE-F in Femoral Bone Marrow Cultures from Normal Mice and Mice Immunized with Killed Group A Streptococcus Vaccine (*M*±*m*)

Bone marrow donor	Month after the end of immunization	Number of nuclear cells per femur, $\times10^6$	CFE-F, $\times10^5$	Content of CFC-F in femoral bone marrow femur
N	1.5-2.0	21.6±1.2	1.3±0.3	283±71
I		23.2±1.2	2.9±0.6	670±112
N	6-8	24.2±1.3	2.1±0.2	413±46
I		24.5±1.8	4.9±0.9	1128±234

TABLE 4. CFE-F in Mouse Bone Marrow Cultures in the Presence of Sera from Immunized and Normal Mice (*M*±*m*)

Serum type	Content of initial serum per culture, μl	CFE-F, per 10^5 explanted cells
-	-	0.4±0.1
Normal	25	1.0±0.2
Immune	25	2.4±0.8
Normal	75	3.6±1.4
Immune	75	6.8±1.8

stromal precursor cell population and suppresses the transplantability and osteogenic activity of SSC. The effects of immunization can be mediated through serum cytokines. These results suggest new ideas about the mechanisms of infection-associated bone tissue defects and can be useful for the search for appropriate therapeutic targets.

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