IMMUNOLOGY AND MICROBIOLOGY

Count of Splenic Stromal Precursor Cells in Mice and Expression of Cytokine Genes in These Cells in Primary Cultures during Different Periods after Immunization of Animals with *S. typhimurium* Antigens

Yu. F. Gorskaya, T. A. Danilova, M. V. Mezentseva, I. M. Shapoval, A. N. Narovlyanskii, and V. G. Nesterenko

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 151, No. 2, pp. 160-163, February, 2011
Original article submitted October 12, 2009

Injection of *S. typhimurium* antigens significantly (9-fold) increased cloning efficiency and, hence, the content of stromal precursor cells in the spleen as soon as after 24 h. These parameters returned to normal by days 6-15 after immunization. Cultured splenocytes collected from immune (but not intact) animals expressed the genes of proinflammatory cytokines IL-1β (on days 1, 6, 15) and IL-6 (on days 1 and 6), TNF-α (on days 6 and 15), and of IFN-α and IL-18 (on days 6 and 15). The expression of IL-4 gene was suppressed on day 6 after immunization, of IL-10 gene on days 1 and 6, of IL-6 gene on day 15. Hence, no signs of immune response suppression by stromal cells were found in this system. The spectrum and dynamics of the expression of pro- and anti-inflammatory cytokine genes in stromal cell cultures from the spleen of immunized mice seemed to correspond to those needed for support of the immune response to *S. typhimurium* antigens, observed in immunized animals. The results indicate possible involvement of stromal cells in the realization of immune response *in vivo*. The increase of stromal precursor cells cloning efficiency in response to antigen injection could not be reproduced *in vitro*: the presence of *S. typhimurium* antigens in primary cultures of intact mouse bone marrow and spleen throughout the entire period of culturing ~20-fold reduced cloning efficiency in cultures.

Key Words: *stromal cells; immune response; cytokine matrix RNA*

Stromal cells (SC) have many functions in a living organism: they are responsible for hemopoietic and lymphoid cell microenvironment, are involved in tissue reparation, act as antigen-presenting cells, and participate in the creation of intercellular matrix.

The content of SC precursors in the lymph nodes of guinea pigs increases more than 30-fold as soon as 24 h after injection of diphtheritic antitoxin [1]. In the bone marrow, the content of these cells increases 2-3-fold after injection of group A streptococcal antigens to animals [3]. This indicates possible involvement of SC in the immune response support.

Laboratory of Immunity Regulation, Laboratory of Microbiology of Latent Infections, Laboratory of Cytokines, N. F. Gamaleya Institute of Epidemiology and Microbiology, Ministry of Health Care and Social Development of the Russian Federation, Moscow, Russia. ***Address for correspondence:*** Uliya.Gorskaya@nearmedic.ru. Yu. F. Gorskaya

However, SC inhibit the lymphocyte immune response to transplantation antigens and mitogens *in vitro* and *in vivo* [13]. On the other hand, mesenchymal stem cells (MSC) express toll-like receptors (TLR) 1, 2, 3, 4, 5, 6, and 9, respond to TLR ligands, and act as antigen-presenting cells in IFN-γ stimulation [12].

The presence of bacterial cell preparations in culture medium suppressed the expression of proinflammatory cytokine IL-1β, IL-2, IL-6, IL-8 genes and stimulated the expression of anti-inflammatory IL-4 cytokine gene in cultured human bone marrow stromal fibroblasts, which was in line with the data on immunosuppressive effect of SC on lymphoid cells. By contrast, the presence of those bacterial cell preparations in primary cultures of mouse bone marrow (containing, apart from stromal fibroblast layer, macrophages and some hemopoietic and lymphoid cells) led to the synthesis of matrix RNA (mRNA) of the cytokines promoting the development of immune response: caused the expression of IL-2, IL-6, and IL-8 genes; IL-1β and IL-6 mRNA appeared in splenic cultures, while IL-4 mRNA disappeared. These data attest to possible involvement of stromal cells in the realization of the immune response *in vivo*.

We studied the effects of animal immunization with *S. typhimurium* antigens on the content of colony forming fibroblast cells (CFC-F) in the spleen during different periods after immunization and on the synthesis of pro- and anti-inflammatory cytokine mRNA in primary cultures of the spleen from immunized mice.

MATERIALS AND METHODS

Experiments were carried out on adult CBA mice (18-20 g). Some animals were intraperitoneally immunized with *S. typhimurium* antigens in a dose of 400 μg/0.4 ml saline. Cell suspensions of intact and immune mouse spleens were prepared as described previously [6]. To some cultures of mouse spleen cells, *S. typhimurium* antigens were added in a concentration of 20 μg/ml culture medium for the entire period of culturing or for 24 h. In order to evaluate the efficiency of fibroblast colony formation (ECF-F), intact mouse spleen cells and cells from immune mice were explanted (5×10^6-1×10^7) into 25-cm^2 flasks on days 1, 6, and 15 after immunization. On days 10-12 these cultures consisted of discrete colonies of stromal fibroblasts with macrophage admixture. The cultures were fixed in ethanol, stained with azur-eosin after Giemsa, and colonies of at least 50 fibroblasts were counted. ECF-F (number of colonies formed after explantation of 10^6 cells) was evaluated by the number of resultant colonies. In order to evaluate the cytokine genes expression in the cultures, intact and immune mouse spleen cells were explanted ($1\text{-}2\times10^7$ per flask). After 8-10 days these cultures consisted of a fibroblast sublayer formed as a result of confluent growth of colonies of SC precursors). The cultures also contained macrophages and some hemopoietic and lymphoid cells. All cultures were incubated at 5% CO_2. The culture medium was then discarded and the expression of cytokine mRNA was evaluated in the cultures.

Activities of mRNA of 11 cytokines, namely IFN (IFN-α and IFN-γ), IL (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18), and TNF-α, were measured in cultured cells by reverse transcription and PCR. RNA was isolated as described previously [5] by acid guanidine thiocyanate phenol chloroform extraction. Reverse transcription and PCR amplification were carried out as described previously [8]. Pairs of primers for the following cytokines were used: IFN-α [8], IL-6, -1β, -2, -4, -10, TNF-α, IFN-γ [14], IL-18 [7], and IL-12 [9]. β-Actin served as positive control [10]. The PCR results were recorded by electrophoresis in 2.5% agarose gel stained with ethidium bromide. Nucleotide sequences were identified using G 1758 electrophoresis marker (Promega).

RESULTS

The morphology of intact and immune mouse splenic cell cultures did not differ from that described previously [1,6]. On days 10-12, the cultures contained discrete colonies (0.1-0.3 cm in diameter) of stromal fibroblasts; the colonies contained macrophage admixture. Injection of *S. typhimurium* antigens caused a significant (9-fold) increase in the content of SC precursors in the spleen as soon as after 24 h (this was in agreement with our previous data [1,3]), followed by gradual normalization of this parameter by days 6-15 after immunization (Table 1). We failed to reproduce the increase in SC ECF-F under conditions of *in vitro* treatment with the antigen. The presence of *S. typhimurium* antigens in intact mouse bone marrow and spleen primary cultures throughout the entire period of culturing 20-fold reduced ECF-F (Table 2). In the culture of guinea pig bone marrow fibroblasts undergoing several passages containing only SC, the antigen *in vitro* had no effect on cell counts in the cultures [4]. These difference were presumably caused by the indirect *in vitro* effect of the antigen on SC mediated in the culture through cells other than stromal ones (macrophages, lymphocytes). Opposite effects of the antigens on stromal tissue *in vitro* and *in vivo* could be caused by violation of the integrity of the organ in which the immune response was unfolding: the stromal tissue either could not adequately react to the antigen and lymphoid cell signals under these conditions or these signals were changed. Normally. IFN-γ,

TABLE 1. Efficiency of Fibroblast Colony Formation in Splenic Cell Cultures from Intact and Immune Mice (*M*±*m*)

Day after immunization	Count of nucleated cells per organ, $\times 10^7$	ECF-F, $\times 10^6$	Number of CFC-F per organ
Intact	16.6±1.0	0.8±0.2	132±24
1	16.6±0.4	7.3±1.0	1207±137
6	16.9±1.2	0.7±0.1	102±6
15	18.0±2.0	0.6±0.2	102±18

IL-2, -4, -8, -10, and -12 mRNA were synthesized in primary cultures of mouse spleen. No expression of IFN-α, IL-1β, -6, -18, and TNF-α mRNA was found (Table 3). This was paralleled by stimulation of proinflammatory cytokines mRNA synthesis in splenic cultures from immune, but not intact animals: IL-1β on days 1, 6, 15, IL-6 on days 1 and 6, IL-18 on days 6 and 15, TNF-α on days 6 and 15, and IFN-α on days 6 and 15. The synthesis of anti-inflammatory cytokine IL-4 was inhibited on day 6 after immunization; during the rest periods it was present in immune and intact spleen cultures. The synthesis of IL-10 mRNA was inhibited on days 1 and 6; IL-6 mRNA disappeared on day 15 after immunization.

Hence, no signs of immune response suppression by splenic SC (the predominant cells in this system) were detected, similarly as in mouse bone marrow [2]. The spectrum and dynamics of the expression of pro- and anti-inflammatory cytokine genes in splenic SC cultures from immunized mice seemed to correspond to those needed for supporting the developing immune response to *S. typhimurium* antigens. A drastic increase in the count of SC precursors in the spleen and appearance of mRNA of the cytokines unfolding the immune response in the primary cultures of splenic cells can result from TLR activation under the effects of bacterial cell preparations. According to some data, stimulation of TLR-3 and -4 in MSC blocked the suppression of T-cell immune response *in vitro* [11]. On the other hand, those results could result from interactions between SC and immunocompetent cells (macrophages, lymphocytes) in the organ expression of proinflammatory cytokine genes was tested and detected in 10-12-day cultures. The spectrum of mRNA varied on days 1-15, depending on the period after animal immunization. Hence, it seems that SC were "programmed" for expression of genes of certain cytokines and that arrest or modification of this "program" were not realized *in vitro*, but only *in vivo*. This fact seems to support the hypothesis according to which the count of SC in the corresponding organs and synthesis of cytokines realized by SC are regulated by stimulated antigens of cells other than stromal (macrophages, lymphocytes). These problems can become the objects of further research.

TABLE 2. Effects of *S. typhimurium* Antigens *In Vitro* on ECF-F in Intact Mouse Bone Marrow and Spleen Cell Cultures (*M*±*m*)*

Organ	ECF-F, $\times 10^6$
Bone marrow, control	22.1±3.0
Bone marrow+*S. typhimurium* antigens *in vitro*	0.1
Spleen, control	0.6±0.1
Spleen+*S. typhimurium* antigens *in vitro*	0

Note. *The antigen was added in a concentration of 20 μg/ml culture medium for the entire period of culturing. Control: cells without antigen.

TABLE 3. Effects of *S. typhimurium* Antigens on Cytokine mRNA Synthesis in Intact and Immune Mouse Spleen Cell Cultures

Day after immunization	IFN-α	IFN-γ	IL-1β	IL-2	IL-4	IL-6	IL-8	IL-10	IL-12	IL-18	TNF-α
Intact	—	+	—	+	+	—	+	+	+	—	—
1	—	+	+↑	+	+	+↑	+	—↓	—↓	—	—
6	+↑	+	+↑	+	—↓	+↑	+	—↓	+	+↑	+↑
15	+↑	+	+↑	+	+	—	+	+	+	+↑	+↑

Note. ↑: appearance of mRNA, ↓: disappearance of mRNA.

On the whole, the data suggest possibly of positive involvement of SC in the realization of immune response.

REFERENCES

1. Yu. F. Gorskaya and A. G. Grosheva, *Immunologiya*, No. 3, 26-29 (1986).
2. Yu. F. Gorskaya, T. A. Danilova, M. V. Mezentseva, *et al.*, *Byull. Eksp. Biol. Med.*, **149**, No. 4, 409-411 (2010).
3. Yu. F. Gorskaya, T. A. Danilova, O. V. Lebedinskaya, and V. G. Nesterenko, *Ibid.*, **140**, No. 7, 77-80 (2005).
4. T. A. Danilova, Yu. F. Gorskaya, E. Yu. Shuklina, *et al.*, *Ibid.*, **118**, No. 11, 489-492 (1994).
5. P. Chomczynski and N. Sacchi, *Anal. Biochem.*, **162**, No. 1, 156-159 (1987).
6. A. J. Friedenstein, N. V. Latzinik, Yu. F. Gorskaya, *et al.*, *Bone Mineral.*, **18**, 199-205 (1992).
7. K. I. Gaede, U. Mamat, M. Schlaak, and J. Müller-Quernhein, *J. Mol. Med.*, **77**, No. 12, 847-852 (1999).
8. C. M. Gelder, P. S. Thomas, D. H. Yates, *et al.*, *Thorax*, **50**, No. 10, 1033-1037 (1995).
9. T. S. Harrison and S. M. Levitz, *J. Immunol.*, **156**, No. 11, 4492-4497 (1996).
10. Y. Lin, M. Zhang, and P. F. Barnes, *Infection Immunity*, **66**, 1121-1126 (1998).
11. F. Liotta, R. Angeli, L. Cosmil, *et al.*, *Stem Cells*, **26**, No. 1, 279-289 (2008).
12. M. Pevsner-Fisher, V. Morad, M. Cohen-Sfady, *et al.*, *Blood*, **109**, No. 4, 1422-1432 (2007).
13. I. Rasmusson, O. Ringen, B. Sundberg, and K. E. Le Blank, *Exp. Cell Res.*, **305**, No. 1, 33-49 (2005).
14. M. Yamamura, K. Uyemura, R. J. Deans, *et al.*, *Science*, **254**, 277-279 (1991).