Effect of Immunization with Polyvinylpyrrolidone on the Counts of Stromal Precursor Cells in the Bone Marrow and Spleen of CBA and CBA/N Mice and Cytokine Gene Expression in Primary Cultures of These Cells

U. F. Gorskaya, T. A. Danilova, M. V. Mezentzeva, M. M. Shapoval, and V. G. Nesterenko

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Injection of polyvinylpyrrolidone (synthetic type 2 T-independent antigen) stimulated the efficiency of clone-forming efficiency and the content of stromal precursor cells in CBA mice in the femoral bone marrow (almost 3-fold) and in the spleen (by 1.7 times) with the peak within 24 h and normalization by day 3 after immunization. The expression of IL-6, IL-8, and TNF-α genes in bone marrow and spleen cultures from immunized animals appeared on day 1 and disappeared on day 3. Hence, stimulation of stromal tissue in response to polyvinylpyrrolidone immunization was significantly less pronounced in comparison with immunization with *S. typhimurium* antigens. The counts of stromal precursor cells in these organs did not increase in CBA/N mice not responding to polyvinylpyrrolidone because they had no xid-mutation in Brutton's tyrosine kinase (Btk) gene, and the proinflammatory cytokine genes expression in primary cultures derived from these animals did not increase either. These data indicated that the degree of stromal tissue stimulation in immunized mice correlated with the immune response intensity. This indicated a close relationship between the stromal tissue and immune system. Stromal tissue seemed to be stimulated not only and not so much through the stromal cell Toll-like receptors, but mainly through interactions of immunocompetent and stromal cells, the former presumably playing the leading role in this process.

Key Words: *stromal cells; immune response; cytokine mRNA*

Stromal cells are responsible for the hemopoietic and lymphoid cell microenvironment, are involved in tissue reparation and in creation of intercellular matrix. Mesenchymal stem cells express Toll-like receptors (TLR) 1, 2, 3, 4, 5, 6, and 9, respond to TLR ligands, and act as antigen-presenting cells under conditions of IFN-γ stimulation [9]. As the stromal precursor cells (CFU-F) of hemopoietic and lymphoid organs provide the specific microenvironment for hemopoietic and lymphoid cell proliferation and differentiation, they are presumably involved in the immune response realization. We have previously shown [1] that injection of *S. typhimurium* bacterial mass to CBA mice causes a significant increase of cloning efficiency (CFE-F) and hence, of CFU-F in the bone marrow (5.5 times) and spleen (9 times) of these animals, with the peak on days 1-3 and subsequent normalization on days 6-15 after immunization. Expression of proinflammatory cytokine IL-1β, IL-6, IL-8, and TNF-α genes emerged in the primary cultures of the bone marrow and spleen from immunized mice as early as 24 after

Laboratory of Immunity Regulation, Laboratory of Latent Infection Microbiology, 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. U. F. Gorskaya

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immunization and persisted during at least 3-15 days after immunization; expression of the anti-inflammatory cytokine IL-4 gene was observed on day 6 after immunization. No expression of this kind was found in primary cultures from intact mice. These data suggest the involvement of stromal cells in the development of immune response, presumably realized by the production of the respective cytokines and attraction of immunocompetent cells into the needed sites and creation of adequate microenvironment for them. It was therefore interesting to find out whether stromal tissue could be stimulated by immunization with non-bacterial antigens and whether the degree of stromal tissue stimulation correlated with the immune response intensity. In order to clear out this question, we used CBA/N mice not responding to type 2 non-T-dependent antigens (in contrast to CBA mice), because they had no CD5+B-1a cells due to xid mutation in the Brutton tyrosine kinase (Btk) gene.

We studied the CFE-F and count of CFU-F in the bone marrow and spleen of CBA and CBA-N mice during different periods after immunization of animals with synthetic type 2 non-T-dependent antigen polyvinylpyrrolidone (PVP) and the changes in the pro- and anti-inflammatory cytokine mRNA spectra in primary cultures of the bone marrow and spleen cells of CBA and CBA/N mice during different periods after PVP immunization of these animals.

MATERIALS AND METHODS

The study was carried out on male CBA mice (18-20 g) from Kryukovo Breeding Center and CBA/N mice from I. I. Metchnikov Institute of Vaccines and Sera. Polyvinylpyrrolidone was injected in a single intraperitoneal dose of 3 μg in 0.4 ml saline. The method for making cell suspensions was described previously [4]. In order to detect CFE-F, the bone marrow or splenic cells were explanted into flasks with bottom area of 25 cm^2 in a dose of $1\text{-}3\times10^6$ (bone marrow) and 5×10^6 (spleen) 1-3 days after immunization. The cells were cultured in α-MEM (Sigma) with 15% FCS (PanEco) in an incubator with 5% CO_2 at 37°C. On days 10-12 the cultures consisted of miscellaneous colonies of stromal fibroblasts with an admixture of macrophages and few hemopoietic and lymphoid cells. The cultures were fixed in ethanol, stained with Azur-eosin, and the colonies containing at least 50 fibroblasts were counted. The CFE-F was evaluated by the number of resultant colonies — that is, the number of colonies formed after explantation of 10^5 cells. The changes in CFE-F and count of CFU-F in the bone marrow and spleen after injection of PVP were evaluated by at least 3 independent experiments in no less than 3 culture flasks per period after immunization. The means with deviations were calculated from the total sum of the results.

In order to evaluate the expression of cytokine genes, bone marrow cells (5×10^6-1×10^7) or splenic cells ($1\text{-}2\times10^7$) of intact and immunized mice were explanted into flasks with bottom area of 25 cm^2. After 8-10 days these cultures consisted of a fibroblast sublayer formed as a result of fusion growth of CFC-F colonies; the cultures also contained macrophages and hemopoietic and lymphoid cells. After the above period the culture medium was discarded and the expression of 11 cytokines mRNA was evaluated in the cells with reverse transcription and PCR. The

TABLE 1. CFE-F in Bone Marrow and Splenic Cell Cultures from CBA and CBA/N Mice Immunized by PVP (*M*±*m*)

Mouse strain	Organ	Day after immunization	Cell count per organ, $\times10^7$	CFE-F, $\times10^5$	Count of CFU-F per organ
CBA	Bone marrow	Control (intact)	0.9±0.2	1.2±0.2	108±24
		1	1.0±0.2	3.0±0.4	300±60
		3	1.0±0.2	1.4±0.1	140±28
	Spleen	Control (intact)	12.4±1.0	0.12±0.02	149±10
		1	12.4±0.3	0.21±0.03	260±63
		3	11.9±1.0	0.20±0.01	205±35
CBA/N	Bone marrow	Control (intact)	0.8±0.2	5.5±0.2	440±110
		1	0.8±0.2	7.1±0.4	568±132
		3	1.0±0.2	5.5±0.1	550±110
	Spleen	Control (intact)	8.8±1.0	0.08±0.02	69±7
		1	8.0±0.4	0.08±0.01	65±2
		3	9.0±1.2	0.08±0.01	72±8

following cytokines were evaluated: IFN-α and IFN-γ, IL-1β, IL-2, 4, 6, 8, 10, 12, 18, and TNF-α. The RNA was isolated by acid guanidine thiocyanate–phenol–chloroform extraction [3]. Reverse transcription and PCR amplification were carried out as described previously [6]. Primer pairs for the following cytokines were used: IFN-α [6], IL-6, IL-8 [9], IL-1β, IL-2, IL-4, IL-10, TNF-α, IFN-γ [13], IL-18, and IL-12 [5]. β-Actin was used as positive control [8]. PCR products were analyzed by electrophoresis in 2.5% agarose gel with ethidium bromide staining. Nucleotide sequences were identified using G 1758 electrophoretic marker (Promega). Activities of mRNA were evaluated by the results of at least 3 independent experiments.

RESULTS

Injection of PVP to CBA mice increased CFE-F and the content of CFU-F by 2.5-2.8 times in the mouse femoral bone marrow and by 1.7 times in the spleen within 24 h after immunization (Table 1).

Bone marrow cells from immune, but not from intact animals, expressed genes of proinflammatory cytokines IL-6, IL-8, and TNF-α on day 1, IL-1β gene on day 3, while the expression of IL-4 gene was suppressed on days 1-3 and of IL-10 gene on day 3. Expression of IL-6 gene in splenic cell cultures from immune animals appeared after 24 h, while expression of IL-4 gene was suppressed (Table 2).

Relatively minor increase in CFU-F count in the bone marrow and particularly in the spleen in comparison with that in response to other antigens (in response to *S. typhimurium* these values increased 5.5 and 9 times, respectively) [1] and narrower spectrum and more rapid disappearance of the expression of pro- and anti-inflammatory cytokine genes in the respective cultures of stromal cells from CBA mice immunized with PVP seemed to reflect slight stimulation of stromal tissue under conditions of weak immune response to PVP. The count of specific antibody-producing cells in CBA mice increased only 2-fold in response to PVP [2].

These hypotheses were confirmed in experiments on CBA/N mice. The levels of CFE-F and of CFU-F in the bone marrow and spleen after injection of PVP to CBA/N mice were virtually the same (Table 1). ECF-F level in the bone marrow (1.3 times) in immune in comparison with intact mice peaked on day 1 after immunization and returned to normal on day 3. No increase of CFE-F was detected in the spleen.

No expression of IL-6, IL-8, and TNF-α genes was detected on days 1-3 in bone marrow and splenic cultures from CBA/N mice immunized with PVP, in contrast to cultures from CBA mice (Table 2). Only suppression of anti-inflammatory IL-4 gene in the

TABLE 2. Effects of PVP Immunization on Cytokine mRNA Synthesis in Primary Cultures of Bone Marrow and Spleen from CBA and CBA/N Mice

Mouse strain	Organ	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
CBA	Bone marrow	Control (intact)	-	+	-	+	+	-	-	+	+	+	-
		1	-	+	-	+	+/-	+	+	+	+	+	-/+
		3	-	+	+/-	+	-	-	-	+	+	+	-
	Spleen	Control (intact)	+	+	+	+	+	-	-	+	+	-	-
		1	+	+	+	+	-	+	-	+	+	-	-
CBA/N	Bone marrow	Control (intact)	-	+	-	+	+	-	-	+	+	+	-
		1	-	+	-	+	+	-	-	+	+	+	-
		3	-	+	-	+	+	-	-	+	+	+	-
	Spleen	Control (intact)	-	+	+	+	+	-	-	+	+	+	-
		1	-	+	+	+	-	-	-	+	+	+	-

Note. "+": mRNA activity; "–": no mRNA; arrow up: mRNA emerged; arrow down: mRNA disappeared.

spleen was found (Table 2). Hence, the absence of immune response to PVP in CBA/N mice caused in fact no response in the stromal tissue: no changes in CPC-F count and the proinflammatory cytokine mRNA synthesis.

These data indicate that stromal tissue stimulation in immune mice correlated with the intensity of immune response to certain antigens.

The presence of bacterial cell preparations in culture medium over 24 h suppressed the expression of proinflammatory cytokine IL-1β, IL-6, IL-8, and IL-2 genes and stimulated the expression of anti-inflammatory cytokine IL-4 gene in continuous cultures of human bone marrow stromal fibroblasts [7], which was in line with the data on the immunosuppressive effect of stromal cells on lymphoid cells [11]. By contrast, the presence of bacterial cells in primary cultures of bone marrow and splenic cells from CBA mice containing, in addition to a layer of stromal fibroblasts, macrophages and some hemopoietic and lymphoid cells led to synthesis of mRNA of cytokines promoting the development of immune response: expression of IL-2, IL-6, and IL-8 was detected; in splenic cultures IL-1β and IL-6 mRNA appeared and IL-4 mRNA disappeared [7]. Stimulation of TLR-4 in human mesenchymal stem cells led to the synthesis of proinflammatory cytokines by these cells in short-term cultures, while stimulation of TLR-3 led to the synthesis of anti-inflammatory cytokines [12]. We can hardly explain our previous results by the fact that in contrast to continuous cultures of human bone marrow fibroblasts, various TLR could be stimulated under the effect of bacterial cell preparations in primary cultures of mouse bone marrow and spleen, as the same bacterial cell preparations were used. In addition, the presence of TLR-4 ligand LPS in human bone marrow mesenchymal stem cell cultures during 24 h suppressed the proinflammatory cytokine genes expression in these cells [7]. Therefore, the expression of genes of cytokines promoting the development of immune response in primary cultures of the bone marrow and spleen was more likely a result of stromal–immunocompetent cell interactions in the cultures. The results of the present study confirm this conclusion. Some data indicate the absence of abnormalities in the stromal tissue of xid mice, because adoptive transfer of lymphocytes from normal mice of the respective strain rapidly and fully restored the response to type 2 non-T-dependent antigens in these animals [10]. Since PVP is a synthetic nonbacterial antigen, our findings suggest that stimulation of stromal tissue cells is realized not only and not so much through TLR, but largely through interactions of immunocompetent and stromal cells, the former ones presumably playing the leading role in this process.

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