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Changes in Production of Cytokines by C57Bl/6J Mouse Spleen during Aggression Provoked by Social Stress

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The effect of aggressive behavior shaped under social stress of various durations on the production of proinflammatory cytokines by splenic cells was examined on C57BL/6J mice. Aggressive mice were characterized by enhanced production of IL-2 and IFN-γ (released by T helper type 1 cells) and reduced secretion of TNF-α, whose major producers are monocytes and macrophages. Elevation of IL-2 and IFN-γ in aggressive mice resulted from enhancement of spontaneous and Con A-stimulated production, the most pronounced effect was demonstrated by the with a longer period (20 days) of victories. In contrast, spontaneous production of TNF-α was similar in control and aggressive mice, although LPS-stimulated production of this cytokine decreased after 10- and 20-day stress. The possible mechanisms of the changes in cytokine production are discussed.

Key Words: *aggressive behavior; cytokines; interleukin-2; interferone-γ; tumor necrosis factor-α*

Clinical and experimental studies revealed a correlation between behavior and immunological responsiveness [2-9,11,14,15]. The experimental models of various types of aggression established that aggressive behavior is accompanied by alterations in functional activity of the immune system. Selection of mice and rats for high and low aggression showed that high-aggressive rodents are characterized by greater immunological responsiveness than the low-aggressive ones [11,14,15]. When formed under a long-term social stress, aggression is accompanied by up-regulation of humoral immune response, redistribution of T-lymphocyte subpopulations, and the changes in composition of various immunocompetent organs [2-5,7,10]. Recent studies showed that cytokines are involved not only in the immunological processes but also in the control of behavioral reactions as well as in pathogenesis of psychic disorders such as depression, schizophrenia, *etc*. Since the cytokines can enter the brain from the peripheral blood and induce synthesis of cerebral cytokines, it seems reasonable to assess production of cytokines by immunocompetent cells during formation of aggressive behavior, which in many cases manifests the psychic disorders accompanied by immunological dysfunction.

Our aim was to analyze the production of proinflammatory cytokines IL-2, IFN-γ, and TNF-α by splenic cells in animals with aggressive behavior resulted from social stress known as a key factor exerting pronounced effects on behavior and functional activity of immune system.

MATERIALS AND METHODS

Experiments were carried out on 2-2.5-month old C57Bl/6J male mice (n=75) weighing 25-30 g. The animals were maintained under vivarium conditions during stable day-night cycle with food and water *ad libitum*. All experimental procedures were carried out in accordance to humanistic principles specified in European Economic Community Directive 86/609/EEC

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and Helsinki Declaration on Protection of Vertebral Animals. All examination protocols were approved by the Biomedical Ethics Committees of SRIPFM and SRIFCI.

Aggressive behavior was formed under the effect of a long-term social stress employing (distant bilateral sensory contact method) [13]. The behavior was tested by the number of attacks against a submissive opponent, the latent time of the first aggressive encounter, and duration of every attack. The study employed the animals that formed the aggressive behavior during daily confrontation tests performed during 10 (group A10) or 20 (group A20) days. The control mice were kept in individual cages for 5 days in order to eliminate the effect of grouping and the agonistic interaction.

The spleen was isolated under sterile conditions. Splenocyte suspension was prepared from the spleens of 3 mice. To assess spontaneous production of IL-2, IFN-γ, and TNF-α, the splenocytes (2×10^6) were cultured in 2-ml wells of a 24-well immunological plates (Linbro) filled with complete culture medium containing RPMI-1640, 10% inactivated FCS (Hyclone), 2 ml L-glutamine (VB VECTOR), 10 mM HEPES buffer (Sigma), and 80 μg/ml gentamicin (Sintez). To stimulate production of IL-2 and IFN-γ, this culture medium was supplemented with mitogen concanavalin A (Con A, 5 μg/ml, Pharmacia), while synthesis of TNF-α was enhanced by adding 10 μg/ml LPS *E.coli* Serotype 055:B5 (Sigma). The cells were cultured for 24, 48, or 72 h.

In each group comprised 21-30 mice, the cytokines were assayed in 7-10 different pools of splenocytes. IL-2, IFN-γ, and TNF-α were quantified in the specimens of splenic culture supernatants according to the product guide employing a Milliplex Luminex 200 multiplexed protein and DNA immunoassay (Merk Millipore), which used magnetic bead-based xMAP technology (Milliplex MAP Mouse Cytokine/Chemokine Panel kit, Millipore).

The data were analyzed statistically using Statistica 10.0 software, Student's *t* test, and single-factor dispersion analysis ANOVA. The results are summarized as m±SEM.

RESULTS

Spontaneous synthesis and mitogen-stimulated production of regulatory cytokine IL-2 by splenocytes were up-regulated in groups A10 and A20 rats with aggressive behavior formed during a long-termed social stress.

In control mice, spontaneous secretion of IL-2 was extremely low, while in groups A10 and A20 rats it was enhanced by 3.1 [F(1,12)=285.31, $p<0.001$] and 5.6 [F(1,12)=127.39, $p<0.001$] times, respectively, in comparison with the control level (Fig. 1). In addition, IL-2 level in A20 group mice was higher than that in mice of A10 group, which had lower score of victories [F(1,12)=35.89, $p<0.001$, Fig. 1]. In 48-h cell cultures derived from the mice of all groups, Con A induced a pronounced elevation of cytokines. However, in the groups A10 [F(1,15)=175.21, $p<0.001$] and A20 [F(1,15)=55.10, $p<0.001$], production of IL-2 was approximately 2 times greater than the control value (Fig. 1).

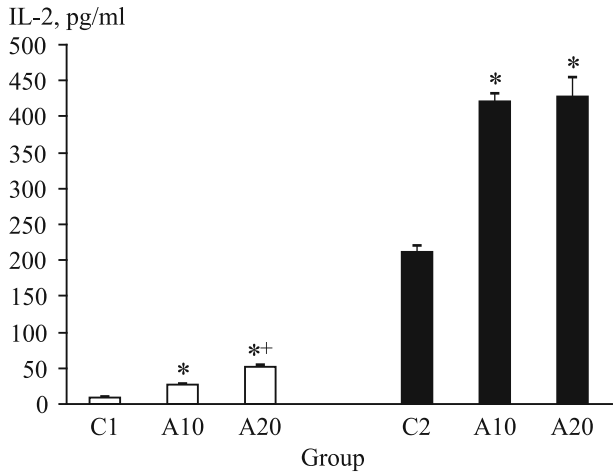

Fig. 1. Production of IL-2 by 48-h incubated splenocytes from control and aggressive mice with various period of victory experience. The open and closed bars correspond to spontaneous and con A-stimulated IL-2 production. C1 and C2 correspond to control mice not conditioned for social confrontations. $p<0.001$ in comparison with *corresponding control; +corresponding A10 group.

The aggressive behavior also enhanced the production of proinflammatory cytokine IFN-γ. In control mice, spontaneous secretion of IFN-γ was low when the splenocytes were incubated for 48 or 72 h without the mitogen (Table 1). In A10 group, spontaneous secretion of IFN-γ exceeded the control level

TABLE 1. Production of IFN-γ (pg/ml) by Splenocytes in Intact and Aggressive Mice with a 20-Day Victory Experience (m±SEM)

Incubation time	Mitogen	Control group	Group A20
48 h	Intact	5.4±0.5 (*n*=7)	43.5±2.1* (*n*=7)
	Con A	4200±400 (*n*=7)	4000±600 (*n*=10)
72 h	Intact	5.2±1.0 (*n*=7)	130±6* (*n*=7)
	Con A	11,800±200 (*n*=7)	13,370±80* (*n*=7)

Note. *$p<0.001$ compared to the control group.

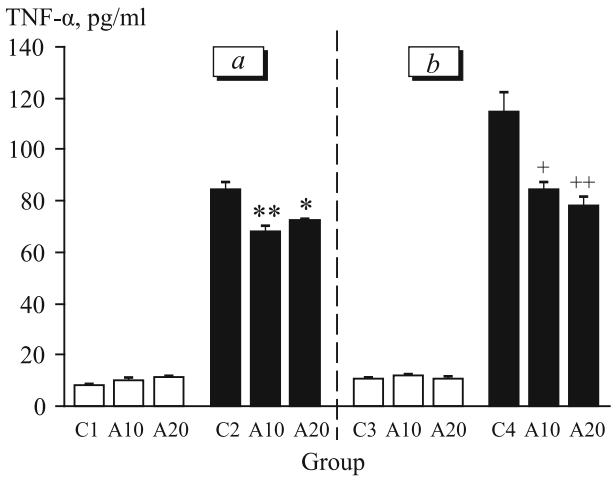

Fig. 2. Production on TNF-α by 24-h (*a*) or 48-h (*b*) incubated splenocytes derived from control and aggressive mice with various period of victory experience. The open and closed bars correspond to spontaneous and LPS-stimulated TNF-α production. C1, C2, C3, and C4 correspond to control mice not conditioned for social confrontations. $^{*}p<0.02$, $^{**}p<0.001$ in comparison with C1 group; $^{+}p<0.02$, $^{++}p<0.001$ in comparison with C4 group.

by 2.7 times (14.4±0.9 and 5.44±0.9 pg/ml, respectively, $p<0.001$). In A20 group, this secretion surpassed the control level and secretion in A10 group by 8 [$F(1,12)=307.85$, $p<0.001$] and 3 [$F(1,12)=146.99$, $p<0.001$] times, respectively (Table 1).

Similarly, spontaneous secretion of IFN-γ was far more intensive (by 24 times) in A20 group than in non-aggressive mice when tested in cultures with longer incubation time of 72 h [$F(1,12)=414.95$, $p<0.001$, Table 1].

In 48-h cultures of both experimental groups, mitogen Con A virtually identically up-regulated IFN-γ synthesis. At the maximum rate of IFN-γ production (in 72-h cultures), the level of this cytokine in supernatant increased even more, and the largest increment in its production was observed in aggressive A20 mice after stimulation of the cells with Con A [$F(1,12)=52.82$, $p<0.001$, Table 1].

In contrast, spontaneous secretion of proinflammatory TNF-α was insignificant, and it was virtually identical in all examined groups (Fig. 2, *a*, *b*). Mitogen LPS significantly up-regulated TNF-α production, the effect being more pronounced in the control group. As a result, the level of TNF-α was lower in the supernatants of Con A-stimulated mice both after 24-h [$F(1,12)=21.83$, $p<0.001$, A10; $F(1,12)=16.1$, $p<0.002$; A20, Fig. 2, *a*] and 48-h incubation [$F(1,13)=14.66$, $p<0.002$, A10; $F(1,12)=17.71$, $p<0.001$, A20, Fig. 2, *b*]. Thus, aggressiveness conditioned by social factors is characterized by the changes in production of proinflammatory cytokines in the spleen. The aggressive mice demonstrated enhanced spontaneous and stimulated production of IL-2 and IFN-γ, which were most pronounced in mice with a long-term (20 days) social stress known to be accompanied by immunostimulation [2-5]. These cytokines are predominantly produced by type 1 helper T cells. Previously we reported an increase in the score of $CD4^+$ T lymphocytes with helper function in the bone marrow of aggressive CBA and C57BI/6J mice [4,10]. In humans, the degree of aggressive behavior positively correlates with the score of $CD4^+$ helper T cells in peripheral blood [9]. In contrast, the level of proinflammatory cytokine TNF-α mostly produced by monocyte-macrophage cells is lower in aggressive animals in comparison with the control ones.

It is a common knowledge that aggression formed in stressful environment is characterized by specific neurochemical cerebral profile with up-regulated activity of dopaminergic system [1,3,4], which plays an important role in immunostimulation [1,10]. Hypothetically, this system exerts diverse effects on immunocompetent cells of T helper and macrophage moiety. It had been shown that enhancement of activity of the dopaminergic system induced by various pharmacological preparations up-regulates the level of $CD4^+$ T lymphocytes in bone marrow [1,10] similarly to what had been observed in aggressive mice [5], but it also down-regulates the functional activity of monocyte-macrophage cells and suppresses production of TNF-α in these cells [12].

The study of peculiarities in the changes of functional activity of the immune system and its regulatory cytokines in aggressive species opens the avenue for a better insight into the mechanisms of pathological forms of social behavior and for the development of effective therapeutic strategies.

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