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Dynamics of Blood Cytokine Concentrations in Rats with Different Behavioral Characteristics after Acute Stress

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Changes in peripheral blood cytokine contents in behaviorally passive and active Wistar rats were assessed at different time points after acute stressing using a restraint model during the dark part of the day. In passive animals, decreases in the concentrations of most of the pro- and anti-inflammatory cytokines studied were most marked immediately and particularly three days after stress. Changes in the cytokine profile of the blood following experimental stress were less marked in behaviorally active individuals: significant decreases from normal were seen only for the proinflammatory cytokine IL-1α and the anti-inflammatory cytokines IL-4 and IL-13. In contrast to passive rats, these changes were more marked in active individuals one day after negative emotional loading. These data illustrate the specific features of the involvement of immunoactive substances in the system controlling the physiological functions and forming individual resistance to the negative sequelae of stress.

Keywords: acute stress, poststress period, blood cytokines, behavior.

The state of stress constitutes the whole set of adaptive-compensatory reactions of mammals to the actions of physical and psychological stress factors leading to impairments in the body's homeostasis. Contemporary conditions are characterized by an accelerated life tempo, urbanization, adynamia, monotony, and information overload. Physiological, psychological, and social studies have shown that the conflict situations which inevitably result from these conditions involve summation of the autonomic and neurological impairments accompanying emotional stress [13, 15].

The classic studies of Selye et al., in 1956 [28] formulated the suggestion that stress is a "general adaptation syndrome" of the body. Stress was regarded as a nonspecific response to the body characterized by a sequence of stages of anxiety, resistance, and exhaustion [27].

Further studies demonstrated that different individuals have different levels of resistance to developing the pathological sequelae of negative emotional stimuli [13, 25]. Behavioral activity in the open field test was found to a predictive criterion for the sensitivity of rats to stress. In terms of survival, active animals were more resistant to stressors than passive individuals [3]. These data suggest that stress is not a nonspecific reaction of all living organisms to extreme situations – reactions induced by the same stressors are different in stress-resistant and stress-susceptible individuals.

This suggestion received support from the results of our previous experiments. Studies using a model of acute and chronic emotional stress showed specific changes in organs serving as stress markers [5] and the gastric mucosa [4, 12] in behaviorally passive and active animals. The characteristics of the behavioral reactions of rats with different initial individual characteristics in stress conditions of different intensities were demonstrated [6, 9]. The specific characteristics of the involvement of biologically active substances, particularly melatonin, in the systems organization of physiological functions in individuals with different predicted stress resistance were identified [8, 11,14].

The pathogenesis of many stress-related diseases involves impairments to the immune system, particularly the cytokine profile of the body's biological media [2, 29, 30].

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Cytokines are polypeptide mediators of intercellular interactions controlling physiological processes both in normal conditions and in pathological states. There is now convincing evidence that stressors of different origins are accompanied by changes in the ratio of pro- and anti-inflammatory cytokines in mammalian tissues [18, 22, 26]. A number of authors believe that these processes are largely due to the stress-inducing balance of Th1 and Th2 cells, which produce immunomodulatory cytokines [16, 19, 24].

Many of the negative consequences of stress only form after the extreme factor has ceased to act on the body. Thus, studies of processes occurring at the early stages of negative emotiogenic stimuli are important in relation to developing new approaches to preventing stress-related pathology. There is interest in understanding the specific development of poststress impairments in individuals with different behavioral parameters characterizing differences in the systems organization of physiological functions in normal conditions and pathology.

Despite existing evidence for the involvement of immune compounds in controlling physiological functions in mammals, data on the specific features of cytokine production and secretion during the poststress period are quite contradictory. The literature lacks data on the different cytokine profiles of biological tissues at different stages after emotiogenic stimulation in individuals with different predicted resistance to identical stressors.

The aim of the present work was to study changes in peripheral blood pro- and anti-inflammatory cytokine profiles produced by monocytes/macrophages and Th1 and Th2 cells in rats with different behavioral activities, at different time points after acute stress.

Methods

Experiments were performed on 75 male Wistar rats weighing 285.0 ± 3.9 g. Experiments were guided by the "Regulations for Studies Using Experimental Animals," approved by a meeting of the Ethics Committee of the Anokhin Research Institute of Normal Physiology (Protocol No. 1, September 3, 2005) and the requirements of the World Society for the Protection of Animals and the European Convention for the Protection of Experimental Animals.

Animals were kept in cages (4–5 individuals per cage) in locations with artificial illumination (light from 09:00 to 21:00, dark from 21:00 to 09:00) at 20–22°C with free access to food and water. After receipt from the supplier, rats were adapted to the laboratory conditions for five days. Animals were subjected to a handling procedure every day – repeated picking up for 15 min – to prevent handling by the experimenter causing stress reactions.

The baseline behavioral characteristics of the rats were identified by testing in an open field for 3 min [3]. The index of activity of the rats was determined as the sum of the numbers of peripheral and central sectors crossed, peripheral and central rearings, and object investigations divided by the sum of the latent periods of the first movement and excursion to the center of the open field. Depending on baseline behavioral parameters in the open field, the rats were divided into passive (n = 37) and active (n = 38) animals, which differed in terms of the mean index of activity (0.46 ± 0.02 and 4.50 ± 0.50, respectively). Four groups of active and four groups of passive animals were then defined, each consisting of 8–10 rats.

Behaviorally, passive and active individuals of experimental groups I and II served as controls. These animals were subjected to the handling procedure and were then placed in their home cages for 12 h before decapitation.

Rats of the other groups were subjected to acute emotional stress. Animals were immobilized in individual plastic tubes of length 16.5 cm and internal diameter 5.5 cm for 12 h during the daytime (21:00 to 09:00). This period was chosen for stress because rats are nocturnal animals, whose activity is maximal during the night. Thus, restriction of the animals' mobility during this period is a very powerful stress factor for these animals. Animals were decapitated immediately (groups III and IV) or at one (groups V and VI) or three (groups VII and VIII) days after experimental stress. Our previous studies showed that changes in a number of physiological metrics in rats in this model of stress were most marked at these time points in the poststress period [7].

Blood collected after decapitation was placed in tubes with a serum diluent (Bio-Plex serum diluent kit), left for 10 min, and then centrifuged at 3000 rpm for 10 min at 4°C (ELMI Multi Centrifuge CM 6M, Latvia). Serum samples were placed in Eppendorf tubes, frozen in liquid nitrogen, and stored in a freezer at –70°C. After thawing samples, peripheral blood serum cytokine profiles were determined using a Bio-Plex instrument (Bio-Rad Laboratories, USA) with a reagent kit for rat cytokine analysis (Bio-Plex Pro™ Rat Cytokine Th1/Th2 Assay) [21]. Levels of the following cytokines were measured: interleukin-1α (IL-1α), IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and granulocyte-macrophage colony-stimulating factor (GM-CSF).

Experimental results were processed using the appropriate statistical and analytical methods run in Statistica 8.0 and Microsoft Office Excel 2007, as well as in the program Bio-Plex (version 4.1). Statistically significant differences in peripheral blood cytokine concentrations in rats of the different experimental groups were evaluated using the Wilcoxon nonparametric T test and the Mann–Whitney U test. The significance level for differences was 5%. Numerical data in tables are given as mean ± standard error of the mean.

Results

Measures of cytokine levels in peripheral blood serum in rats of the different experimental groups in standard conditions and after 12 h of restraint stress are shown in Tables 1 and 2.

At baseline, between-group differences in serum cytokine concentrations in animals with different behavioral parameters in the open field test were seen for IL-10, IL-12,

TABLE 1. Peripheral Blood Serum Cytokine Concentrations in Behaviorally Passive Rats of Different Experimental Groups (pg/ml, M ± m)

Cytokines	Control (intact)	12-h immobilization stress → analysis		
		immediately after stress	1 day after stress	3 days after stress
IL-1α	355.58 ± 76.04	249.56 ± 67.91	375.19 ± 69.65	258.16 ± 75.68
IL-1β	868.84 ± 168.58	460.22 ± 92.75**	1292.72 ± 221.15* °°	679.80 ± 183.19+
IL-2	4236.81 ± 634.56	3129.31 ± 802.22	4102.69 ± 635.87	2092.35 ± 735.46** +
IL-4	377.81 ± 73.35	324.94 ± 70.36	320.40 ± 86.52	229.07 ± 55.10*
IL-5	481.13 ± 50.87	393.35 ± 31.16*	454.84 ± 32.22	359.58 ± 50.92* +
IL-6	2477.76 ± 636.07	2273.64 ± 670.37	1857.20 ± 404.60	1543.10 ± 175.03*
IL-10	1684.11 ± 158.13	1010.02 ± 253.70**	1028.69 ± 280.98**	1128.80 ± 310.82*
IL-12	157.14 ± 27.13	66.32 ± 19.21**	112.08 ± 16.81* °	102.70 ± 37.49
IL-13	197.06 ± 42.99	134.13 ± 41.40	203.67 ± 26.51	159.95 ± 50.35
IFN-γ	625.26 ± 90.63	403.91 ± 93.13*	478.44 ± 94.98	351.35 ± 51.68**
TNF-α	147.71 ± 33.14	112.56 ± 20.18	138.03 ± 35.20	119.34 ± 32.11
GM-CSF	145.76 ± 41.39	116.29 ± 40.48	128.90 ± 29.22	29.75 ± 8.50** °° ++

Here and inTable 2: *$p < 0.05$ and **$p < 0.01$ compared with controls (intact rats); °$p < 0.05$ and °°$p < 0.01$ compared with values immediately after stress; +$p < 0.05$, ++$p < 0.01$ compared with values one day after stress.

TABLE 2. Peripheral Blood Serum Cytokine Concentrations in Behaviorally Active Rats of Different Experimental Groups (pg/ml, M ± m)

Cytokines	Control (intact)	12-h immobilization stress → analysis		
		immediately after stress	1 day after stress	3 days after stress
IL-1α	436.01 ± 61.79	300.11 ± 72.88	261.54 ± 46.08*	365.53 ± 80.19
IL-1β	1008.01 ± 142.80	960.34 ± 191.55xx	732.15 ± 244.81x	809.71 ± 210.55
IL-2	4042.92 ± 536.97	3641.35 ± 587.68	2904.79 ± 706.81	2887.85 ± 788.91
IL-4	377.29 ± 46.80	315.95 ± 61.91	231.12 ± 70.95*	325.20 ± 92.93
IL-5	401.01 ± 46.07	383.16 ± 58.05	361.69 ± 25.21x	413.34 ± 73.25
IL-6	2228.33 ± 258.69	1671.41 ± 371.76	1951.94 ± 565.69	2139.87 ± 486.53
IL-10	957.15 ± 306.38x	1133.41 ± 162.70	919.02 ± 184.20	1305.04 ± 168.61+
IL-12	88.38 ± 19.08x	89.85 ± 18.61	96.02 ± 36.24	122.33 ± 50.62
IL-13	176.24 ± 32.70	154.80 ± 26.16	102.08 ± 33.64* xx	137.21 ± 23.41
IFN-γ	489.53 ± 87.44	464.82 ± 105.12	537.78 ± 88.88	459.76 ± 134.31
TNF-α	82.31 ± 16.60x	96.08 ± 36.79	75.46 ± 14.79x	82.05 ± 13.59
GM-CSF	130.20 ± 27.02	79.61 ± 19.27	96.67 ± 25.38	124.38 ± 30.80xx

x$p < 0.05$ and xx$p < 0.01$ compared with passive rats.

and TNF-α. Peripheral blood levels of these cytokines in behaviorally active individuals were 1.8 times lower than in passive rats ($p < 0.05$). There were no statistically significant differences in the contents of the other cytokines studied in animals of these groups in control conditions.

Blood IL-1α concentrations in behaviorally active rats subjected to negative emotional stress gradually decreased, reaching minimal levels one day after stress (to 40.0% below control, $p < 0.05$). Cytokine levels in these animals increased at three days after stress, but remained 16.2% below the levels in unstressed individuals. Immobilization of passive rats during the dark period of the day was accompanied by wavelike oscillations in IL-1α concentrations as compared with the control level: a decrease immediately after stress (by 29.8%), followed by an increase to normal at one day after stress, and then a repeat decrease on observation day 3 (by 27.4%). However, these changes did not reach statistical significance.

Peripheral blood IL-1β concentrations in in animals which were active in the open field test were almost no different from control levels immediately after restraint stress, but decreased slightly by days 1 and 3 of the study (by 27.4% and 19.7%, respectively; not statistically significant). In contrast to these individuals, levels of this cytokine in passive animals decreased by 47.0% immediately after stress ($p < 0.01$ compared with unstressed rats). At one day, rats of this group showed a sharp increase in the blood IL-1β concentration to a level 48.8% greater than baseline ($p < 0.05$).

At this time point, the IL-1β content in behaviorally passive animals was 1.8 times greater than that in active individuals ($p < 0.05$). By observation day 3, the cytokine level in these individuals decreased ($p < 0.05$ compared with day 1) and was somewhat lower than baseline.

The blood IL-2 concentration in behaviorally active rats decreased slightly during the poststress period as compared with the baseline level; however, these changes were not statistically significant. In passive animals, wavelike changes in the IL-2 content immediately and one day after restraint stress were followed by a sharp decrease in its concentration by observation day 3 (by 50.6% compared with controls, $p < 0.01$).

The peripheral blood serum IL-4 concentration in active animals subjected to stress showed a gradual decrease, reaching a minimum one day after stress (38.7% below control, $p < 0.05$). By study day 3, the cytokine content in these rats increased, but remained 13.8% below the level in unstressed individuals. The blood IL-4 level in animals which were passive in the open field test showed almost no change immediately and one day after restraint stress, but decreased by 39.4% by observation day 3 ($p < 0.05$ compared with baseline).

The serum IL-5 level in behaviorally active rats showed a minor decrease immediately and one day after acute stress but increased to a level indistinguishable from baseline by study day 3. Restraint of passive individuals during the dark period of the day led to wavelike changes in IL-5 concentrations from the control level, with a decrease immediately after stress (by 18.7%, $p < 0.05$) and a subsequent increase to normal at one day after stress and a repeat drop on observation day 3 (by 25.3%, $p < 0.05$). It should be noted that the IL-5 content one day after experimental stress in behaviorally active animals was 1.3 times greater than that in active rats ($p < 0.05$).

The peripheral blood IL-6 concentration in animals which were active in the open field test showed some decrease immediately after stress (by 25.0% compared with controls, not statistically significant), but gradually increasing during the subsequent period and returning to normal by observation day 3. The content of this cytokine in passive individuals decreased progressively during the poststress period and reached a minimum at three days after restraint stress (by 37.7% compared with unstressed animals, $p < 0.05$).

Blood IL-10 concentrations in active rats were characterized by statistically insignificant wavelike changes during the poststress period. In behaviorally passive animals, the content of this cytokine showed a marked decrease immediately after the end of restraint stress and hardly changed by observation days 1 and 3 (40.0% ($p < 0.01$), 38.9% ($p < 0.01$), and 33.0% ($p < 0.05$), respectively, below baseline).

The serum IL-2 level in active rats showed essentially no change immediately or one day after stress, but increased slightly by observation day 3 (by 38.4% compared with controls; not significantly different). The content of this cytokine in individuals which were passive in the open field test decreased immediately after restraint stress – by 57.8% compared with that in unstressed animals ($p < 0.01$). At one day after stress, the blood IL-12 concentration in these animals increased from the preceding time point ($p < 0.05$) but remained 28.7% lower than baseline ($p < 0.05$). There were no significant differences in the peripheral blood IL-12 level in passive rats three days after restraint (compared with observation day 1).

The peripheral blood IL-13 concentration in behaviorally active rats decreased slightly immediately after stress and reached a minimum by observation day 1 (by 12.2% and 42.1% ($p < 0.05$) below control, respectively). The level of this cytokine in active individuals increased the three days after restraint but remained lower than baseline. Restraint of passive rats during the dark part of the day was accompanied by wavelike oscillations in the IL-13 concentration as compared with controls: there was a decrease immediately after stress (by 31.9%) followed by an increase to normal at one day after stress and a repeat decrease on observation day 3 (by 18.8%). However, these changes were not statistically significant. The blood IL-13 content in passive animals one day after restraint was 2.0 times greater than that in active individuals ($p < 0.01$).

Serum IFN-γ levels in active rats barely changed during the three observation days after acute stress. As compared with baseline, the concentration of this cytokine in animals which were passive in the open field test decreased immediately after restraint stress (by 35.4%, $p < 0.05$), showed some increase by observation day 1, and again decreased at poststress day 3 (by 43.8%, $p < 0.01$).

The peripheral blood TNF-α concentration in rats with different behavioral parameters subjected to acute restraint stress was characterized by statistically insignificant wavelike changes. It should be noted that the content of this cytokine at all poststress time points in behaviorally active animals was lower than that in passive individuals (most marked one day after experimental stress, when the level of reduced by a factor of 1.8 ($p < 0.05$)).

The serum GM-CSF concentration in rats which were active in the open field test decreased immediately after stress (by 38.9% compared with controls, not statistically significant), but increased gradually during the subsequent period to reach normal by observation day 3. The level of this cytokine in passive animals showed almost no change immediately and one day after stress, but decreased by 79.6% by observation day 3 ($p < 0.01$ compared with baseline).

Discussion

The studies reported here show that rats with different behavioral parameters in the open field test had particular peripheral blood cytokine profiles. The serum concentrations of the anti-inflammatory cytokine IL-10 and the proinflammatory cytokines IL-12 and TNF-α in behaviorally active individuals were significantly lower than those in passive animals. This demonstrates a difference in the tension of im-

mune reactions in control conditions in individuals with different predicted sensitivities to negative emotiogenic stimuli. These results supplement current data on the existence of a link between the intensity of immune processes in the tissues and behavioral activity in mammals. In particular, in normal conditions, elimination of foreign substances from the abdominal cavity to the regional lymph nodes and wall of the small intestine, reflecting the functional activity of the immune system, was found to be more marked in behaviorally passive rats than in active individuals [1].

Our studies showed that behaviorally passive animals were characterized by complex changes in the serum levels of the cytokines studied at different stages of the poststress period. Immobilization of rats during the dark part of the day was found to lead to wavelike changes in the blood concentrations of most proinflammatory cytokines, synthesized by Th1 cells (IL-2, IL-12, IFN-γ, and TNF-α) and monocytes/macrophages (IL-1): decreases occurred immediately after stress and were followed by increases at one day after stress and repeat decreases on observation day 3. These changes were seen for anti-inflammatory cytokines IL-5 and IL-13, which are produced by Th2 cells. Peripheral blood GM-CSF and IL-6 contents in passive animals, which decreased gradually immediately and one day after restraint stress, reached a minimum on observation day 3. At this time after acute stress, passive individuals also showed low peripheral blood concentrations of anti-inflammatory cytokines IL-4 and IL-10, produced by Th2 cells. Thus, changes in the cytokine profile in behaviorally passive rats, consisting of decreases in the concentrations of pro- and anti-inflammatory cytokines, were most marked immediate and especially three days after stress.

Our experiments demonstrated that 12-h restraint of behaviorally active animals was accompanied by less marked changes in peripheral blood cytokine concentrations than those seen in passive individuals. The contents of most cytokines in rats of this group were significantly decreased at different stages of the poststress period. Statistically significant decreases in these metrics from normal values were seen only for the proinflammatory cytokine IL-1α and the anti-inflammatory cytokines IL-4 and IL-13. In contrast to passive animals, these changes in active individuals were more marked at one day after negative emotional stimulation.

These data supplement the results of our previous studies. Restraint stress for 1 h with simultaneous subthreshold electrocutaneous stimulation was followed immediately by a decrease in the serum concentration of the proinflammatory cytokine IL-1β in behaviorally passive animals, though there was an increase in active individuals. The blood content of the anti-inflammatory cytokine IL-4 in rats with different behavioral characteristics hardly changed in these conditions [10]. IL-1β, one of the key mediators of the acute phase of the stress reaction, triggers the secretion of a whole cascade of other cytokines in the body and has an activating influence on the hypothalamo-hypophyseal-adrenal complex [17, 20, 23]. It can be suggested that passive individuals with predicted susceptibility to stress demonstrated similar changes independently of the type of stress, i.e., there was a decrease in the level of this cytokine immediately after negative emotiogenic stimulation. In contrast to animals of this group, the nature of oscillations in IL-1β concentrations in behaviorally active rats predicted to be resistant to the negative sequelae of stress are significantly dependent on the stress model used – with increases in immobilization with electrocutaneous stimulation but only minor changes in restraint during the dark part of the day. The absence of marked change in blood IL-1β levels in active individuals after nocturnal restraint stress evidently makes some contribution to the relative stability of the levels of the other cytokines studied here during the poststress period.

The data presented here supplement the results of our previous studies of stress in rats in the same model of stress – 12-h immobilization during the dark period of the day. The diurnal rhythms of behavior and heat production in animals changed markedly during the first two days, though there was a trend to normalization on day 3 of the poststress period [7]. Summarizing these data, we can conclude that oscillations in metabolic and behavioral parameters reflect a shift in the body's endogenous biological rhythms in experimental stress, accompanied by changes at the biochemical level, i.e., blood immune status.

Thus, animals with initially different behavioral parameters were characterized by peripheral blood cytokine profiles at different stages after acute emotional stress. These data illustrate the specific features of the involvement of immunoactive substances in the systems regulation of physiological functions and the formation of individual resistance of the negative sequelae of emotiogenic stimulation.

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