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Long-lasting Effects of Social Stress on Peritoneal Inflammation in Some Strains of Mice*

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Adult male mice were kept for one week either one or four animals per cage. Some were maintained under the same social conditions for an additional 9 days (controls); their counterparts were either grouped (4 per cage) or isolated (1 per cage). Changes in housing conditions caused a significant increase of plasma corticosterone measured 30 minutes after separation or grouping of SWISS, C57C3H, and BALB/c but not of C57BL/6 mice. Peritoneal inflammation was induced by i.p. zymosan injection on day 9 after changes in housing conditions when corticosterone was again at its initial level in each group. Peritonitis-connected pain symptoms, exudatory PMN numbers, and cytokine (IL-1β and MPC-1) and corticosterone levels were compared between animals living in stable social conditions with those shifted 9 days earlier from separation to the group or *vice versa*. These factors were unaffected by social stress in C57BL/6 mice and in SWISS animals transferred from the group to isolation. In all other instances at least two parameters were significantly different in the post-stressed and control animals, being either enhanced or inhibited. In conclusion, social stress had long-term consequences on the course of inflammation in three out of four investigated strains of mice.

Key words: Stress, housing, corticosterone, peritonitis, pain, IL-1β, MCP-1.

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Experimental peritonitis is a convenient model for testing pro- and anti-inflammatory drugs (e.g. DOHERTY *et al*. 1987; CHADZINSKA *et al*. 1999; KOLACZKOWSKA *et al*. 2002; PLYTYCZ & NATORSKA 2002), as it is easy to retrieve from the inflamed peritoneal cavity the exudatory fluid containing inflammation-related cells and soluble factors which can be subjected to precise qualitative and quantitative analysis. The course of inflammation is species- and strain-specific and varies according to the age and sex of experimental animals as well as according to the kind and dose of stimulus used (MENASZEK *et al*. 1999; SCISLOWSKA-CZARNECKA *et al*. 2000; STANKIEWICZ *et al*. 2001; PLYTYCZ & NATORSKA 2002; KOLACZKOWSKA *et al*. 2003). In the case of ectothermic animals, kinetics of inflammation is also season-dependent and affected by the ambient temperature (MENASZEK *et al*. 1999). The typical time-course of inflammation may be modified by factors connected with the experimental procedure. For example, sterile pre-injection of physiological saline or even sterile puncture or handling of experimental animals may modify the early stages of the subsequently evoked peritoneal inflammation (PLYTYCZ *et al*. 1998; NATORSKA *et al*. 2001). It was also shown that mice delivered by various suppliers differed in their reaction to the same pro-inflammatory stimulus (KOLACZKOWSKA *et al*. 2003). Besides minor genetic differences between animals bred by various suppliers, this discrepancy may be caused by social stress connected with transportation and changes in housing conditions. This could explain the variations between various sets of experiments performed by the same investigators on laboratory strains of mice carefully matched according to age

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and gender under the same laboratory conditions. As a rule numerous mice share the same large cages during transportation. Only after arrival are constant numbers of cage-mates distributed to the final cages and allowed to adapt for at least one week to strictly controlled light, humidity, and housing conditions in the new animal facilities (CHADZINSKA *et al*. 1999, 2001; KOLACZKOWSKA *et al*. 2001a, b).

For present investigations we used C57C3H males from the breeding colony in our Department and SWISS, BALB/c and C57BL/6 male mice from commercial suppliers. The two former strains are commonly used by our team (e.g. PLYTYCZ *et al*. 1998; CHADZINSKA *et al*. 2001, 2003; STANKIEWICZ *et al*. 2001) while BALB/c and C57BL/6 animals were selected because they are known to be, respectively, stress-sensitive and stress-resistant (MOYNIHAN *et al*. 1994; LU *et al*. 1998; SHANKS & KUSNECOV 1998). The aim of experiments was to check if the grouping or isolation of mice has indirect effects on inflammation-related factors. Therefore peritoneal inflammation was induced only on day 9 after changes of housing conditions. The results show that a 9-day period of adaptation is too short to restore homeostasis disrupted by stress due to changes in housing conditions in stress-sensitive strains of mice.

Material and Methods

Adult male mice from SWISS, BALB/c and C57BL/6 strains were purchased from commercial suppliers (Breeding of Laboratory Animals, Warszawa, Poland and Collegium Medicum, Kraków, Poland). C57C3H mice were reared in the Institute of Zoology (Jagiellonian University, Kraków, Poland) by random mating of individuals from F2 generation of C57BL/6xC3HFeB/J (kindly provided by Professor W. Wiktor-Jędrzejczak from Warszawa). The animals were kept in 20x13x18 cm cages in a room with controlled temperature (22^{o}C) and lighting (lights on 8:00-20:00) conditions, one or four mice per cage. Food (mouse laboratory chow) and water were available *ad libitum*. Animals were 6-8 weeks of age (25-30 g body weights) at the start of investigations. The experiments were conducted according to license no. 16/OP/2001 from the Local Ethical Committee.

For stress due to isolation, animals were housed under strictly controlled laboratory conditions for 7 days in constant groups of four mice per cage (GG groups). Then mice from some cages were separated and further maintained one animal per cage, forming GS groups while their control counterparts continued to live in 4-individual groups (GG). Such a number of animals per cage was recommended as optimal by PENG *et al*. (1989).

For stress due to grouping, animals were housed under strictly controlled laboratory conditions for 7 days separately, one animal per cage (SS groups). Then four mice of the same strain were put together forming SG groups, while their control counterparts continued to live separately (SS).

Peritonitis was induced on day 9 after animal separation or grouping (i.e. in GS and SG mice) and in their respective controls (GG and SS). At 10:00 am, peritoneal inflammation was induced as described previously (KOLACZKOWSKA *et al.* 2001b, CHADZINSKA *et al*. 2001, 2003). In brief, 0.5 ml of 2% Zymosan A solution (Sigma, London, UK) was injected i.p. Animals were killed by cervical dislocation at 10:00 (time 0 hrs) or at selected time points after induction of peritonitis, mainly at 14:00 (time 4 hrs).

The peritoneal cavity was lavaged with one ml of saline and exudatory fluid was retrieved. Peritoneal leukocytes (PTL) including polymorphonuclear cells (PMN) were stained with Turk solution and counted in a hemocytometer. Cytokine levels (IL-1β and MCP-1) in lavaged fluids were evaluated using ELISA, according to the manufacturer's procedure (BioSource, Camarillo, USA).

Nociceptive activity was tested using the writhing model (DOHERTY *et al*. 1987). Characteristic body writhes (consisting of a contraction of the abdominal muscles together with a stretching of hind limbs) were counted during five-minute intervals for each mouse. The number of these writhes was high during the first half-an-hour after zymosan injection, while thereafter these symptoms were sporadic only. For statistical comparisons the cumulative number of writhes occurring between 0 and 35 min after zymosan injection was used.

Corticosterone concentration was measured in blood plasma by radioimmunoassay according to the manufacturer's procedure (ICN, Costa Mesa, USA).

For statistical analysis, Student's *t*-test and 2-way analysis of variance (ANOVA) followed by post hoc Tukey test were used, with differences considered statistically significant at $P<0.05$.

Results

Corticosterone level in blood plasma measured 30 minutes after animal isolation or grouping was significantly higher than that before shifting (at time 0) in SWISS, C57C3H, and BALB/c while its increase was insignificant in C57BL/6 mice. A stress-induced increase of corticosterone was most pronounced in the grouped SWISS males that be-

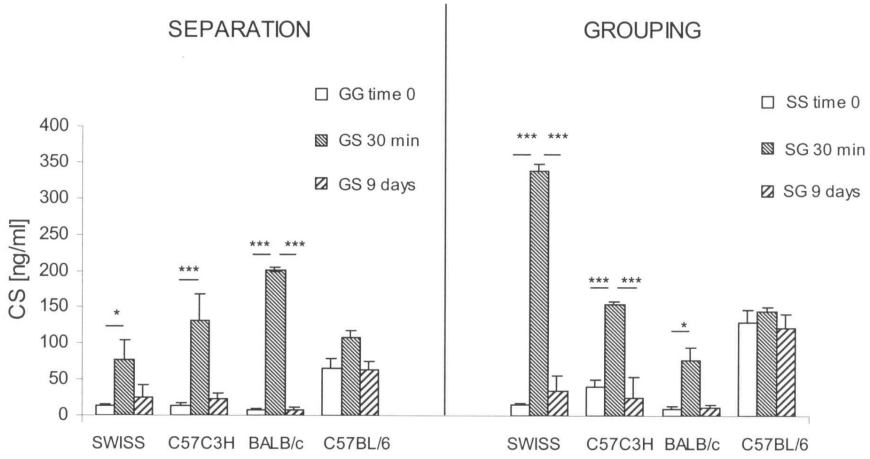

Fig. 1. Effects of social stress due to separation (left) or grouping (right) on corticosterone (CS) levels in males from four strains of mice. CS level before changes in housing conditions (time 0) in mice kept 4 per cage (GG, left) or one per cage (SS, right) and 30 min or 9 days after shift from group to separation (GS, left) or from separation to group (SG, right). Values are means (±SE) from 4-8 samples; P<0.05 (*), or P<0.001 (***).

came aggressive, in contrast to isolated SWISS males, where corticosterone increase was significant but not so profound. On day 9 after changes of housing conditions corticosterone was at the control level in each group of mice (Fig. 1).

In all strains the level of corticosterone was significantly increased during the inflammation (e.g. in SWISS males see Fig. 2a).

The general course of inflammation in SWISS males was similar to that described previously in SWISS (CHADZINSKA *et al*. 2001), C57C3H (NATORSKA *et al*. 2003), BALB/c and C57BL/6 mice (KOLACZKOWSKA *et al*. 2001b, 2003). The number of exudatory polymorphonuclear leukocytes (PMNs) (Fig. 2b), the levels of proinflammatory cytokines IL-1β and MCP-1 in peritoneal fluid (Fig. 2c) were significantly increased at 4 hours after zymosan injection thus these factors and this time point were selected for further analysis. Animals with peritonitis (but not their intact counterparts) exhibited characteristic body writhes considered to be pain symptoms (RIBEIRO *et al*. 2000; DOHERTY *et al*. 1987). The number of these writhes was high during the first half-an-hour after zymosan injection, while thereafter these symptoms were sporadic only (Fig. 2d).

Body writhes (pain symptoms) were compared between the animals subjected to the social stress of separation or grouping 9 days earlier with their unstressed controls. In several instances the course of pain symptoms was almost identical in shifted animals and their and non-shifted controls (e.g. in GG/GS groups of SWISS and C57BL/6 males) (Fig. 3). In other instances the course of pain symptoms was significantly different in stressed and unstressed males of the same strain, which was especially striking in C57C3H males, where stress

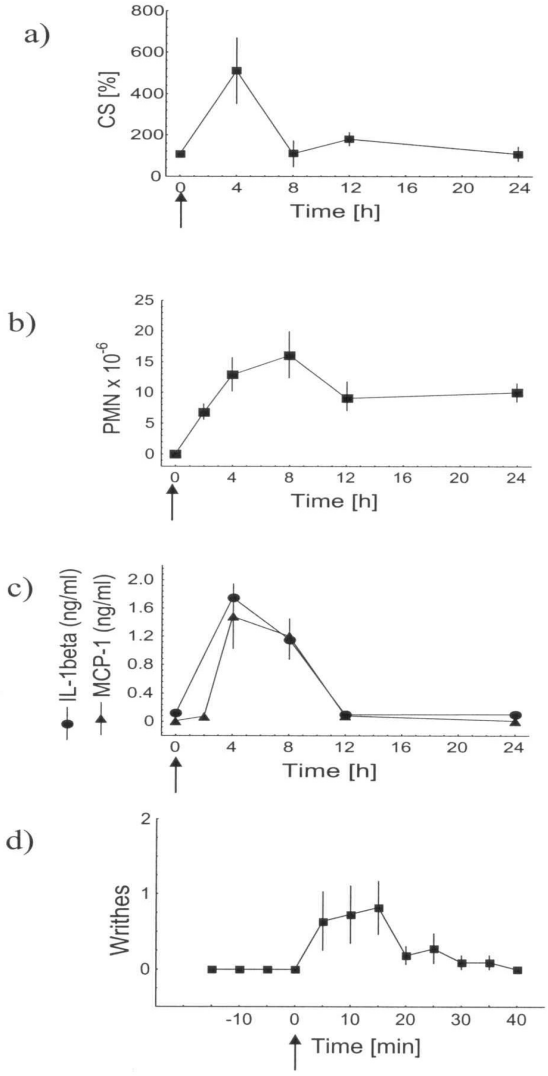

Fig. 2. Kinetics of some inflammation-related parameters at selected time points after intraperitoneal zymosan injection (arrows, time 0) in Swiss males, 8-12 mice /group. (a) blood plasma corticosterone (CS) – percent of that at time 0; (b) numbers of peritoneal polymorphonuclear cells (PMN); (c) levels of cytokine IL-1beta and chemokine MCP-1 in peritoneal fluid; (d) body writhes. Values are means (±SE).

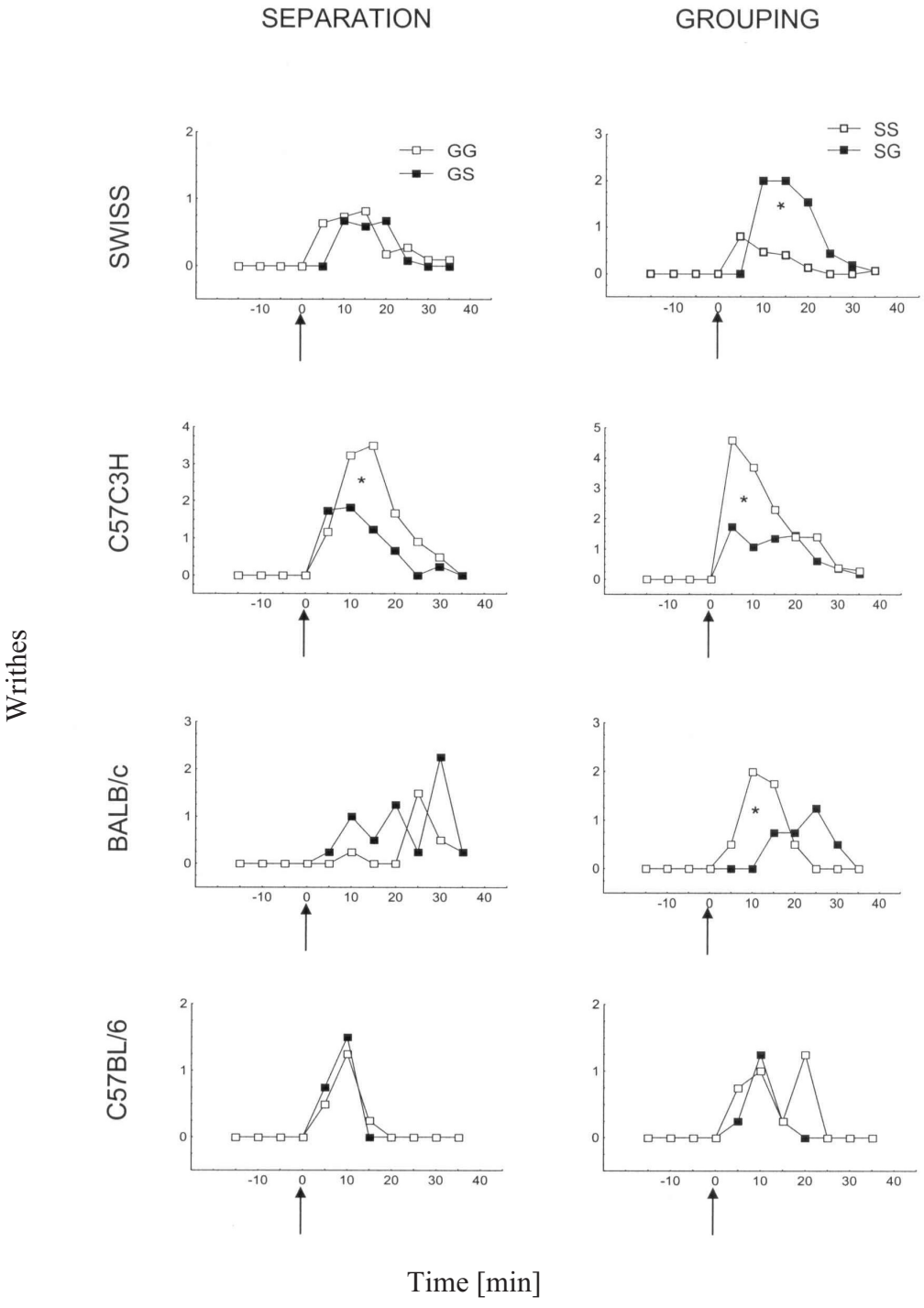

Fig. 3. Effects of social stress due to separation (left panel) or grouping (right panel) on the numbers of body writhes after i.p. zymosan injection (arrows) in several strains of mice. Animals housed 4 per cage (GG), one per cage (SS) or shifted from group to separation (GS) or from separation to group (SG). Values are counts in 5-minute intervals; $P<0.05$ (*).

of both isolation and grouping inhibited pain symptoms, while stress of grouping enhanced them in SWISS males (Fig. 3).

Similar results were obtained by statistical analysis of cumulative numbers of body writhes counted during 35 minutes after zymosan injection by Student's *t*-test (Fig. 4a). In comparison with unstressed controls, the numbers of body writhes were significantly increased in the grouped SWISS and isolated BALB/c mice, while they were significantly decreased in C57C3H males, both after isolation or grouping (Fig. 4a). The same pattern of changes concerned the number of exudatory PMNs retrieved from the peritoneal cavity 4 hours after zymosan injection. Additionally, the

number of PMNs was significantly increased in the grouped BALB/c mice (Fig. 4b). In comparison with control samples from animals kept in stable housing conditions, the level of IL-1β measured 4 hours after zymosan injection was significantly increased in isolated C57C3H mice, but it was significantly decreased in grouped males of BALB/c males (Fig. 4c). The level of MCP-1 was significantly decreased in grouped BALB/c only (Fig. 4d). The level of plasma corticosterone was significantly increased in isolated BALB/c males but significantly decreased in grouped C57C3H males (Fig. 4e).

Discussion

The results of experiments presented here showed that stressful changes of housing conditions had long-term consequences on males of three out of four investigated mouse strains as they modified the course of peritonitis induced by zymosan injection 9 days later.

Stress is a state of threatened homeostasis provoked by a psychological, environmental, or physiologic stressor (CHROUSOS & GOLD 1992). A stressor can be defined as an internal or external stimulus that activates the hypothalamic-pituitary-

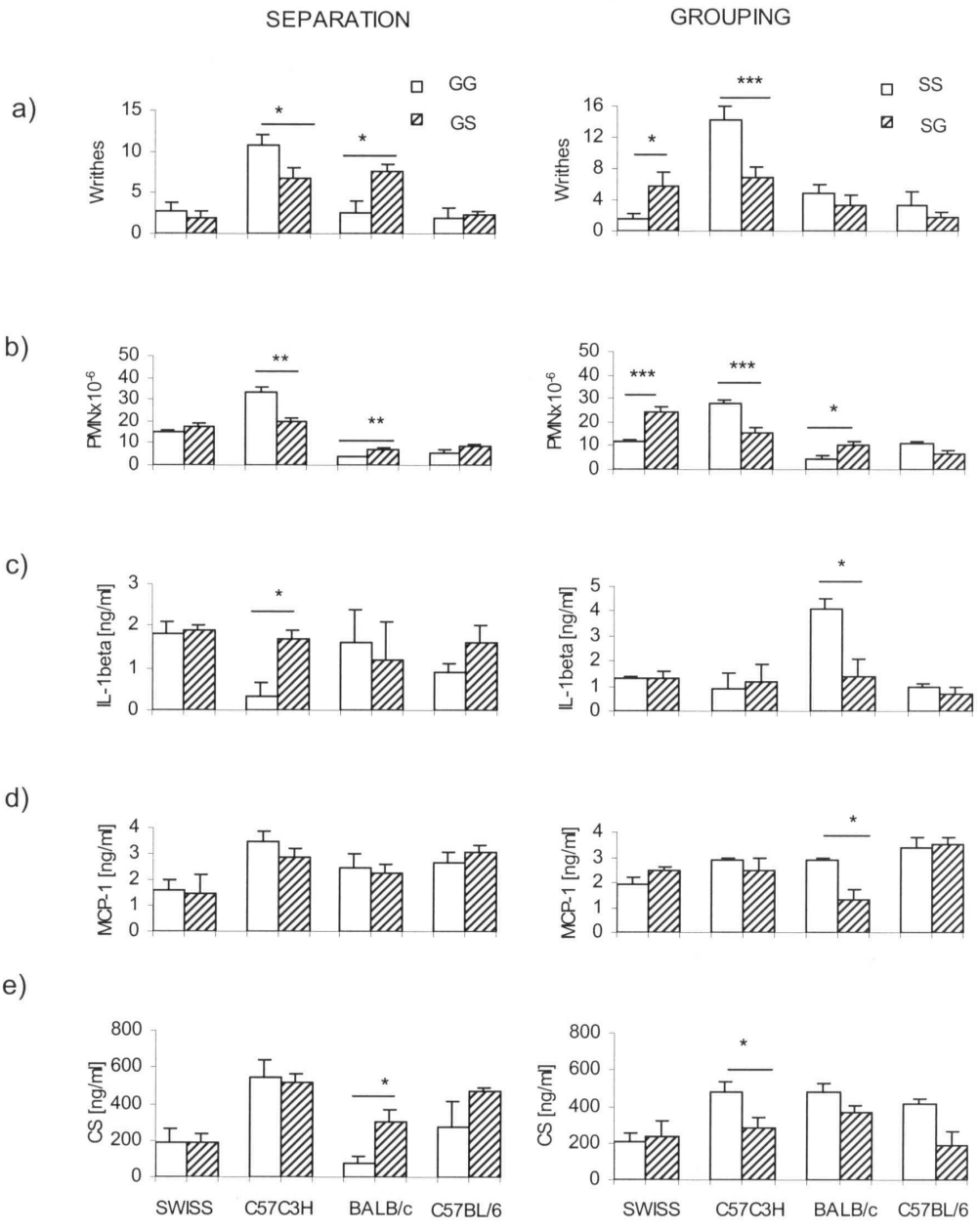

Fig. 4. Effects of social stress due to separation (left panel) or grouping (right panel) on some inflammation-related parameters 4 hours after i.p. zymosan injection in different strains of mice. (a) body writhes; (b) numbers of peritoneal polymorphonuclear cells (PMN); (c) levels of cytokine IL-1beta in peritoneal fluid; (d) levels of chemokine MCP-1 in peritoneal fluid; (e) level of corticosterone (CS) in blood plasma. Animals housed 4 per cage (GG), one per cage (SS) or shifted from group to separation (GS) or from separation to group (SG). Values are means (±SE). $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***).

adrenal axis (HPA) resulting in elevated levels of glucocorticoids (MAIER & WATKINS 1998). The grouping or isolation of laboratory rodents belongs to the animal paradigms of social stress (BLANCHARD *et al*. 2001). In the experiments presented here, social stress increased the corticosterone level recorded 30 minutes after shifting of male mice from isolation to the group or *vice versa*. This was negligible and statistically insignificant in C57BL/6 males, most pronounced and highly significant in freshly grouped SWISS males (exhibiting fighting behaviour), and moderate but statistically significant in isolated SWISS males as well as in two other strains (C57C3H and BALB/c) subjected to isolation or grouping. In all instances corticosterone was at its initial level 9 days later, and only then was experimental inflammation induced. Despite the low level of corticosterone, the course of inflammation was modified in most instances which indicates that body homeostasis of stressed animals was not yet fully restored. This was not surprising as recent studies have shown that regulation of the immune system by glucocorticoids is only one part of an extensive regulatory network between the central nervous system, neuroendocrine system, and immune system (WEBSTER *et al*. 2002; BLACK 2002; PADGETT & GLASER 2003).

Inflammation-related factors were compared between males kept in the standard groups of 4 animals per cage (GG) with their counterparts separated 9 days earlier (GS). On the other hand, comparisons were made between males kept during the whole experimental period separately (SS) with animals grouped 4 per cage (SG) 9 days before induction of inflammation. Notably, an increased level of blood plasma corticosterone was recorded during peritonitis. A similar phenomenon was revealed in the case of cortisol during peritonitis in the goldfish (SCISLOWSKA-CZARNECKA 2001). The increased level of glucocorticoids during inflammatory processes indicates that inflammation may be considered as a stress-reaction itself (PLYTYCZ & SELJELID 1995, 2002).

At least in the time points selected here, symptoms of inflammation were unaffected by the isolation or grouping of C57BL/6 mice and by the isolation of SWISS males. The former corresponds with a lack of a significant corticosterone increase after changing of housing conditions of C57BL/6 males. In all other instances, namely in the grouped SWISS mice and in both isolated and grouped C57C3H and BALB/c males, at least two out of five investigated inflammation-related parameters were significantly different in the post-stressed and control animals being either enhanced or inhibited. However, the changes reported here did not exhibit any consistent pattern (Table 1).

Stress-resistance of C57BL/6 immune factors reported here corresponds with the results obtained by MOYNIHAN *et al*. (1994) who developed an elegant model of a purely psychological stress, namely exposition of mice to odors produced by footshock stressed conspecific animals. They showed that C57BL animals were unaffected when exposed to such a stressor while under the same conditions BALB/c mice had increased antibody titers and IL-4 levels in comparison with the recipients of odors from unstressed mice. It was proved that endogenous opioids are responsible for the stress odor-induced immune deviations in BALB/c mice (MOYNIHAN *et al*. 2000). The immune system of BALB/c mice was also affected by housing conditions as WU *et al*. (2000) revealed a reduced proliferative response of splenocytes and inhibited splenic NK activity in isolated males, as compared with group-housed mice.

Various effects of housing conditions on SWISS CD-1 males were recorded by BARTOLOMUCCI *et al*. (2003). They showed that adult males housed individually for 3 weeks showed an increased basal level of corticosterone, decreased proliferation of splenocytes and reduced production of IL-2 (as

Table 1

Effects of social stress due to separation or grouping on several inflammation-related parameters in different strains of mice

	SWISS		C57C3H		BALB/c		C57BL/6	
	GG/GS	SS/SG	GG/GS	SS/SG	GG/GS	SS/SG	GG/GS	SS/SG
Writhes	≈	↑	↓	↓	↑	≈	≈	≈
PMN	≈	↑	↓	↓	↑	↑	≈	≈
IL-1b	≈	≈	↑	≈	≈	↓	≈	≈
MCP-1	≈	≈	≈	≈	≈	↓	≈	≈
CS	≈	≈	≈	↓	↑	≈	≈	≈

Values in animals separated (GS) were compared with their controls kept 4 per cage (GG) while values in animals grouped (SG) were compared with their controls kept separately (SS). Values were similar (≈), significantly higher (↑), or significantly lower (↓) than their respective controls. PMN – polymorphonuclear cells, IL-1β – interleukine1β, MCP-1 – monocyte chemoattractant protein 1, CS – corticosterone.

compared to group-housed siblings), without changes in IL-10, IFN-γ and β-endorphin. SHANKS *et al*. (1994) recorded that the shift of CD-1 males from group to individual housing resulted in a suppression of antibody production to antigen inoculated after five days of isolation but not earlier. SALVIN *et al*. (1990) showed the activity of macrophages and antibody response of C3H/HeJ males housed individually were significantly stronger than those in mice housed five per cage.

A complex variability of stress-dependent fluctuations of immune factors in mice was recorded by LU *et al*. (1998). They investigated stress-induced changes in ConA or LPS-induced splenocyte proliferation, natural killer cell activity, and macrophage functioning in males of BALB/c, C57BL/6ByJ, and outbred CD-1 mice subjected to psychogenic (exposure to a rat as a predator) or neurogenic footshock stress. It turned out that stress-induced immune variations are not only strain-specific but also vary between investigated immune factors and between kinds of stressors. These authors concluded that analyses of stress effects on immune functioning need to consider the species and strain of investigated animals, the particular immune parameter being examined and the nature of stressors. Our preliminary results indicate gender-dependent differences in response to changes in housing conditions (SCISLOWSKA-CZARNECKA *et al.* 2000). It is worth mentioning that the inter-strain differences may be obscured by the time of sampling owing to strain-specific daily fluctuations of immune factors (KOLACZKOWSKA *et al.* 2001a).

Laboratory rats are also sensitive to housing conditions. BALDWIN *et al*. (1995) showed that individual housing of Sprague-Dawley rats for 3.5 weeks elicited an increase in basal corticosterone, peripheral blood leukocytes, and blood glucose levels. Long-lasting effects of psychological threat (under protection against physical trauma) were recorded by CAROBREZ *et al*. (2002) in Wistar rats exposed to an aggressive intruder of a wild-type strain, as one week later the threatened rats showed an increased risk of septic shock after i.p. LPS injection. In contrast to laboratory rodents, other species seem to be less sensitive to housing conditions. For example, single and group housed rabbits did not differ in physiological and immune measurements (WHARY *et al.* 1993). Also calves seem to habituate easily to relocation (VEISSIER *et al.* 2001).

Also other manipulations routinely used in laboratory practice may be stressful for animals and affect their immunity. Simple handling (i.e. picking up by the tail and holding gently in the palm of the hand for two minutes daily) of Balb/c female mice every day for two weeks prior to immunization suppressed antibody response (MOYNIHAN *et al.* 1990). Similar treatment increased pulmonary metastases of alveolar carcinoma (BRENNER *et al.* 1990). A significant increase in NK cell activity was recorded in BALB/c females following handling (HALE *et al*. 2003). Intraperitoneal injection of saline suppressed antibody response in C3H/HeJ male mice (MOYNIHAN *et al.* 1989). In mice stressed by handling and/or sterile injections, inhibition of peritoneal inflammation was recorded (NATORSKA *et al*. 2001, PLYTYCZ *et al*. 1998). Sterile injection-induced inhibition of inflammation was reversed by morphine treatment, which implicates the involvement of opioid receptors (PLYTYCZ *et al*. 1998). Inhibition of immunity was also induced in animals stressed by cold or isolation. In these experiments the reduced number of inflammatory macrophages was compensated by their increased activity measured by nitric oxide production (SHAPIRA *et al.* 2000). The most striking example of stress-induced immunosuppression is that elicited by purely psychological stressors such as odors from stressed conspecific animals (MOYNIHAN *et al.* 2000; ZALCMAN *et al.* 1991).

Several studies address the effects of stress on the immune response (for reviews see PLYTYCZ & SELJELID 2002; BLACK 2002; WEBSTER *et al*. 2002; PADGETT & GLASER 2003; MOYNIHAN 2003). The main purpose of the present paper is to warn investigators that apparently minute laboratory changes and everyday routines might have long-lasting significant effects on the results of experiments on laboratory animals.

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