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Effects of Midazolam on the Activity of Phagocytosis in Mice Submitted to Surgical Stress

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FREIRE-GARABAL, M., M. J. NÚÑEZ, J. L. BALBOA, J. GONZÁLEZ-BAHILLO AND A. BELMONTE. *Effects of midazolam on the activity of phagocytosis in mice submitted to surgical stress.* PHARMACOL BIOCHEM BEHAV **46**(3) 605–608, 1993.—Mice submitted to surgical stress and treated with chronic midazolam (1 mg/kg) showed a reduction in stress-induced suppression of the in vitro and in vivo activity of phagocytosis, both measured using the zymosan-particle uptake method and the carbon clearance test, respectively.

Midazolam Carbon clearance test Macrophages Mice Phagocytosis Stress Zymosan test

ADVERSE effects of stress on various kinds of cell-mediated immune events, such as the activity of phagocytosis, have been previously reported. Macrophages from stressed mice showed a depressed functional activity under in vivo and in vitro conditions (21,23), which was postulated to have resulted, among other factors, from elevated plasma corticosterone levels.

Midazolam is a central and peripheral benzodiazepine agonist with well-known anxiolytic properties. In previous studies (14), we observed an attenuatory effect of midazolam against adverse effects of stress on several immune events. Mice submitted to surgical stress and treated with chronic midazolam (1 mg/kg) showed a reduction in stress-induced suppression of thymus and spleen cellularity, and in peripheral lymphocyte population. The blastogenic response of spleen lymphoid cells was also assessed, and midazolam was found to partially reduce the suppressive effect of surgery. Nevertheless, there are few data on the effects of midazolam on the activity of phagocytosis during or after stress. To further elucidate this latter interaction, in the present paper we study the effect of midazolam on the in vivo and in vitro phagocytosis in mice submitted to surgical stress.

METHOD

Mice

Female mice (8 weeks old at the beginning of the experiment) of the BALB/c strain (Interfauna Ibérica S.A., Barcelona, Spain) were used. They were housed, 7 days before experiments, four per cage in an aseptic chamber kept between 21 and 22°C and maintained on an alternating 12L : 12D cycle. Sterilized food (Panlab Diet A.03) and water were given ad lib.

Procedure

Mice were randomly divided into five groups: group A, operated and injected with placebo; group B, operated and injected with midazolam; group C, sham-operated and injected with placebo; group D, sham-operated and injected with midazolam; group E, unstimulated controls. At 24, 48, and 72 h after surgery, two lots of six mice per group were used to determine the in vivo and in vitro activity of phagocytosis, respectively.

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Induction of Surgical Stress

The mice were subjected to a 1-cm incision in the dorsal surface under ether anesthesia in aseptic conditions. The inhalational anesthetic was delivered with a total carrier gas flow of 5 l/min into a 40-l chamber, which allowed gas to exit at one end. Anesthetic depth was maintained at a level at which the mice exhibited only sleeping behavior. An occasional staggering journey by a mouse followed by somnolence was considered acceptable (15). At the end of surgery, a 5 l/min oxygen flow was maintained through the chamber until arousal was noted. Surgery was always performed at 5:00 p.m. Unstimulated controls were exposed only to the normal activity of the animal room.

In Vitro Phagocytosis of Macrophages

Macrophages were collected by washing the peritoneal cavity with ice-cold PBS (0.1 M, pH 7.3). The peritoneal cells were washed twice with RPMI culture medium (GIBCO Laboratories, Gran Island, NY) by centrifugation (1000 rpm, 5 min, 0°C) and finally resuspended in an adequate volume of RPMI medium supplemented with 10% heat-inactivated fetal calf serum (FCS, GIBCO) to give a concentration of 1×106 nucleated cells per ml. Aliquots (1 ml) of this suspension were seeded in 35-mm petri dishes and incubated at 37°C in a humidified 5% CO_2 and 95% air incubator. Three hours later, nonadherent cells were washed off, and 1 ml fresh medium containing 10% (FCS) was added. Zymosan was added to give 5×106 particles/ml and incubation was continued. Thirty minutes later, particle uptake was measured by light microscopy. Cells containing three or more zymosan particles were counted as phagocytic (21).

In Vivo Phagocytosis of Macrophages

Phagocytic activity was estimated by using the carbon clearance test. Carbon suspension (Pelikan c11/1432a) was centrifuged at 5000 rpm for 15 min, and the supernatant was diluted threefold with sterile 1.5% gelatin saline to bring the carbon concentration to about 30 mg/ml. Diluted carbon suspension was injected at 0.1 ml/10 g body weight into the tail vein of mice. After 0.5 and 10 min of injection, a blood sample (0.05 ml) was collected by puncturing the retro-orbital venous plexus. Blood was hemolyzed by the addition of 1 ml 0.1% Na_2CO_3 solution, followed by measurement of its optical density at 600 nm. Phagocytic index, K, was derived from the following equation (21):

$$K\frac{1}{t_{10} - t_{0.5}} log \frac{C_{0.5}}{C_{10}}$$

where $C_{0.5}$ and C_{10} express the carbon concentration at time $t_{0.5}$ and t_{10}, respectively.

Drug Treatments

The drug tested was midazolam (Roche S.A., Madrid, Spain), which was intramuscularly (IM) injected in a dose of 1 mg/kg (13). Control stressed mice were IM injected with vehicle as placebo. Volume of all injections was 1 ml/kg. Drugs were injected 1 h before and 12, 24, and 48 h after surgery.

Statistical Analysis

Statistical analysis was performed using the Student's t-test analysis. Differences were considered to be significant when the probability (p) value was < 0.05.

RESULTS

Table 1 shows the results of in vitro phagocytic activity of peritoneal macrophages cultured with zymosan particles for 30 min. The number of macrophages containing zymosan particles was reduced to 35% of the control in mice injected with vehicle and sacrificed 24 h after surgery. By contrast, this decrease was lower (60% of the control) in mice injected with midazolam (differences significant at $p < 0.01$). Values in sham-operated mice injected with saline were also reduced in comparison with those of the controls, although differences were smaller than those of operated mice. Sham-operated mice injected with midazolam also had greater numbers in comparison to those injected with placebo.

As shown in Fig. 1, carbon clearance test also showed a stress-induced reduction of in vivo phagocytic activity. Similarly, the decrease in the carbon clearance rate was lower in mice injected with midazolam (37% of the control) compared with mice injected with vehicle (12% of the control). Differences between controls and sham-operated mice injected with

TABLE 1

EFFECTS OF SURGICAL STRESS AND MIDAZOLAM ON IN VITRO PHAGOCYTOSIS BY PERITONEAL MACROPHAGES

	Percent Particle (zymosan) Uptake of 100 Cells			
Unoperated controls	56.4 ± 8.17			
	Laparotomy		Sham Surgery	
Hours After Surgery	Placebo	Midazolam	Placebo	Midazolam
24	19.7 ± 3.5	28.9 ± 4.5	30.3 ± 6.0	41.5 ± 3.1
48	20.3 ± 4.1	34.2 ± 6.8	39.5 ± 2.7	47.9 ± 7.0
72	29.8 ± 1.3	39.8 ± 5.0	42.1 ± 6.1	53.2 ± 7.2

BALB/c mice were subjected to an incision in the dorsal surface. Macrophages were collected by washing the peritoneal cavity of these mice and cultured in RPMI-1640 medium containing 10% FCS with 5×10^6 zymosan particles for 30 min. Particle uptake was measured as described in the Method section. The results represent the mean ± SD of six animals. Differences between placebo and midazolam were significant at $p < 0.05$. Differences between surgery and sham-surgery mice were significant at $p < 0.05$.

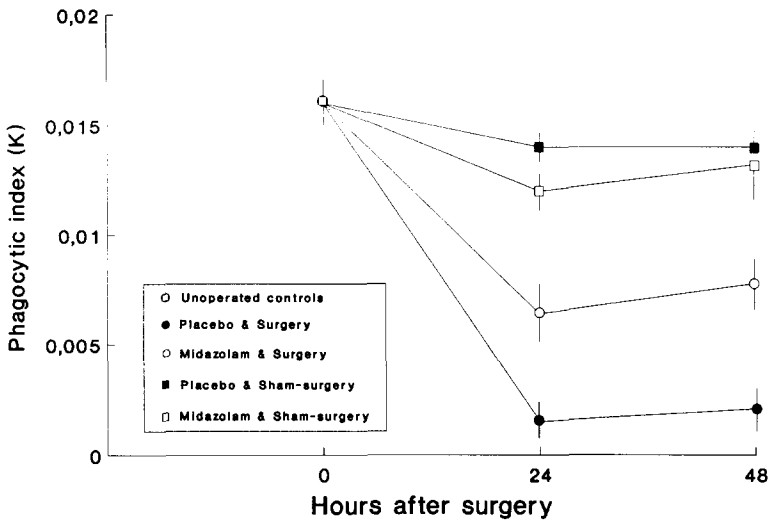

FIG. 1. Effect of midazolam on the carbon clearance activity in mice submitted to surgical stress. Carbon suspension (30 mg/ml) was injected at 0.1 ml/10 g body weight into the tail vein of mice. After 0.5 and 10 min of injection, a blood sample (0.05 ml) was collected by puncturing the retro-orbital venous plexus. Phagocytic index (K) was calculated as described in the Method section. The results represent the mean ± SD of six animals. Differences between placebo and midazolam in operated mice were significant at $p < 0.01$. Differences between unstimulated controls and sham-operated mice injected with placebo were significant at $p < 0.05$ at 24 h.

saline were only significant ($p < 0.05$) at time 24 h. No differences were appreciated between unstimulated controls and sham-operated mice injected with midazolam.

DISCUSSION

Our results show that both anesthesia and laparotomy caused a decrease in the phagocytic activity in mice. Both the in vivo and in vitro studies showed a sharp fall in the uptake of zymosan particles and in the carbon clearance rate, respectively, in mice. Nevertheless, this suppressive effect was more intense in mice subjected to laparotomy. These data are in good agreement with previous reports in the field of psychoimmunology showing a suppressive effect of different schedules of stress (16,21,23) on the phagocytosis in mice. On the other hand, the inhibitory effects of anesthesia and surgery on in vivo and in vitro phagocytic activity were partially recovered by the administration of midazolam.

As for the mechanism of action of stress on the phagocytosis, it is not yet clear. Okimura et al. (21) found that restraint stress caused suppression of in vivo phagocytic activity that was recovered by the infusion of serum from normal mice. In vitro phagocytic activity of peritoneal macrophages was also significantly suppressed in stressed mice. These results indicated that depression of phagocytic activity was due to both the impairment of the functions of macrophages and the changes in serum components of stressed mice.

A molecular basis for bidirectional communication between the immune and neuroendocrine systems has been described (1,4–12,25,28). At least one of the neuroendocrine responses to stress, such as the increase in plasma corticosterone levels, via ACTH secretion, has been usually involved in stress regulation of the phagocytic activity of macrophages (23,27). In our previous investigations, we also observed that surgical stress induced an increase in ACTH levels proportional to the decrease in the activity of macrophages. Furthermore, ACTH from the pituitary gland, and even IR-ACTH from lymphocyte origin, has a direct inhibitory effect on functional capacities of macrophages. About 47% of unstimulated peritoneal macrophages express ACTH receptors (26).

Midazolam is a benzodiazepine agonist with well-known anxiolytic properties. Benzodiazepines have been found to reduce the stimulatory effect of stress on ACTH and corticosterone levels that were attributed to the activation of GABA-linked benzodiazepine receptors in the CNS (2,18).

A second aspect is the existence of a peripheral benzodiazepine receptor (PBR) with high affinity on immune cells. Benzodiazepines have been found to exert immunomodulating activities by binding on a specific receptor on macrophages (29,30). For example, benzodiazepines have been found to affect the production of microbicidal and tumoricidal reactive oxygen species by macrophages.

Midazolam has central and peripheral BZD receptor agonist properties. So, its effects on the activity of phagocytosis may be either direct (on the macrophage) or indirect (e.g., on ACTH). In our study, we observed a protective effect of midazolam on the in vivo and in vitro activity of phagocytosis, which is overly strong in favor of an indirect effect affecting the neuroendocrine response to stress.

In a previous report, we observed a correlation between the immunoenhancing effects of midazolam and the reduction of plasmatic ACTH concentrations (14). Nevertheless, adrenalectomized mice showed a lower pattern of immunosuppression in response to stress. So, this leads us to believe that other neuropeptides and neurotransmitters may be involved in the immunosuppressive response to stress and in the action of benzodiazepines (3,12,16,17,19,24).

Nevertheless, the large number of interactions at molecular, cellular, and functional levels between the nervous system and the immune system characterizing the operational

compositions and expressions of the neuroimmune network make complex the isolation of the pathways in which benzodiazepines may be involved in the regulation of the immune response to stress. Moreover, biological significance and health relatedness of these immunological effects should be assessed.

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