Moroccan Journal of Biology 07-2008/N 4-5

Preliminary Studies of Immunological, Gastric and Behavioural Changes in Mice Following Stressor Exposure

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Abstract

The aim of this study is to investigate the effects of experimental stress (unavoidable footshock) on immune, gastric and behavioural responses at different intervals after 1, 3 and 5 days of immunization: Immune responses were analyzed in terms of both humoral and cellular immunity: serum antibody concentration was determined by ELISA techniques. The cellular immunity was investigated by enumerating both white blood cells subpopulations (WBC) (lymphocytes, neutrophiles, basophiles, monocytes and eosinophiles) by using a coulter counter and subtypes of peripheral blood lymphocytes, T helper/inducer CD4, T suppressor/cytotoxic (CD8), and natural killer (NK) cells by flow cytometry analysis. Behavioural responses were analyzed using two tests: staircase and light/dark box tests. Finally, stress effect on gastric mucous was evaluated by histological cut techniques and microscopical analysis. The results indicate that application of experimental stress for 1 day after immunization decreased all subtypes of leukocytes except eosinophiles which seem to be increased after stress exposure. Furthermore, the decrease of lymphocytes was noted only in T CD4 number, whereas T CD8 lymphocytes number was not changed after stress exposure. In contrast, the number of NK cells increased in stressed mice; mice exposed to experimental stress show a decrease of their exploratory, locomotory and postural behaviours. These changes were accompanied by an increase of gastric erosions, which be explained partly by a decrease of neutrophiles number into circulation and their infiltration in the mucosal gastric. We can suggest the existence of simultaneous variation of behavioural, gastric and immune responses after stress exposure in mice.

Key words: ***Stress, Cellular immunity, humoral immunity, behaviour, gastric erosions.***

Introduction

The study (Selye, 1936) has reported that some physiological changes occurred in animals that are exposed to a wide variety of stressors. Since that time, many different restraint procedures have been used to show that the most them are associated with a variety of both central and peripheral changes indicative of stress, including atrophy of the thymus and spleen (Selye, 1936) gastric ulcers (Simpson *et al*. 1975, Vincent *et al*. 1977), reduced gastrointestinal transit (Applebaum & Holtzman, 1985), hypothermia (Murakami *et al*., 1985) and accelerated noradrenalin and dopamine turnover in many brain regions (Glavin, 1983).

Other researches have shown that stressful events and acute psychological stress can modify the functioning of the immune system (Esterling & Rabin 1987,

Kappel *et al*. 1991, Kusnecov *et al*. 1992, Dobbs *et al*. 1993) and alter components of cellular immunity in humans (Bachen *et al*. 1992, Kiecolt-Glaser *et al*. 1992, Zakowski *et al* 1992). It had been demonstrated that acute exposure to stressors may induce a suppression of immune activity (Dantzer & Kelly, 1989). Furthermore, a series of experiments carried out by authors of the study (Zalcman *et al*., 1988) revealed that mice exposure to footshock, 72 h after sheep red blood cells (SRBC) inoculation provoked a marked suppression of the splenic plaque forming cell (PFC) response and antibody titers. In accordance with our current study, the work of (Edwards *et al*., 2006) noted that acute stress exposure can enhance the antibody response in humans prior to influenza vaccination.

Other works have shown that human psychological stress caused a significant increase of T CD8, NK cells and total WBC (Naliboff *et al*. 1991, Herbert *et al*. 1994, Mills *et al*. 1995). However, the studies performed by (Bachen *et al*. 1992, Herbert *et al*. 1994) demonstrated that the number of T helper/inducer CD4 and CD19 lymphocytes do not change after psychological stress in humans while (Marsland *et al*., 1997) showed that the percentage and the absolute numbers of T CD8 and NK lymphocytes increased after stress, whereas T CD4 and CD19 lymphocytes decreased. However, it's reported that chronic stress appears to induce a suppression of the immune response, whereas immune activation and suppression have been associated with acute stress (Maddock & Pariante, 2001).

In animals, experimental stress (novelty, forced swimming, restriction of environmental space) had been shown to induce changes in various behavioural parameters, including decreases in general locomotor activity and exploratory behaviour (Misslin *et al*. 1982, Aloisi *et al*. 1997, Mitsushima *et al*. 1998). Other works indicated that stressful experiences influence the vulnerability to gastric ulcer (Ludwig-William & Lipkis 1969, Yano & Harada 1973, Vincent *et al*. 1977, Honda *et al*. 1994, Redei *et al*. 1994). This include increases in acid and pepsin, decreases in mucus, alterations in adrenal steroids and catecholamines, hypotension followed by ischemic damage to the gastric mucosa and alterations in gut prostaglandin synthesis. These events could be generally controlled by the central nervous system (Hernandez, 1986).

The main aims of this study are (i) to investigate the immune response in mice exposed to experimental stress, (ii) to evaluate locomotor and exploratory behavioural responses in stressed mice (iii) to examine the influence of experimental stress on gastric mucous membrane in mice and to verify the existence of simultaneous variation of behavioural, gastric and immune responses of mice.

Materials and methods

Animals

Male Swiss mice (centre d'élevage R. Janvier, France), weighing between 38 g and 44 g, 12 weeks of age and group-housed (five per box) in polypropylene boxes containing food and water ad libitum. Mice were acclimatized to laboratory conditions for two weeks before being used. The light cycle was automatically controlled (08 h at 20h00) and room temperature adjusted to maintain 21 ± 1°C.

Immunization

Mice received an intraperitoneal administration "i.p", (10^6) of sheep red blood cells according to study of Laudenslager *et al*. (1988).

Procedure of stress

Each group of five mice was kept in the shock boxes measuring "27.5 x 24 x 28.5", connected to a shock generator (leitica, LE 100-26 shocker, France) and subjected to 300 unavoidable shocks (2 sec/shock, 150 µA, 9s inter-shock interval) according to study of Zalcman *et al*. (1988).

These shocks were applied at days 1, 3 and 5 following antigen inoculation. The control animals were not shocked; all the animals were sacrificed 24 hours after stress application.

Time which separated antigen inoculation and application of shocks were varied (1, 3 and 5 days), but time separating stress exposure and animal sacrifice were kept constant 24 h; previous results have demonstrated that sacrifice of animals immediately after stressor exposure did not show any effect on immune, behavioural and gastric responses compared to animals sacrificed 24 hours after stress (unpublished data).

1) Immunological study

Blood collection

Mice were anesthetized with pentobarbital "i.p" injection (Sanofi, France), 6% diluted at 1/10 in the NaCl 0.9 %. Blood was collected in heparinised tubes by heart puncture at 1, 3 and 5 days following immunization.

- White blood cells counts (WBC)

Total concentrations of WBC in peripheral blood were determined by using a coulter counter.

- Determination of serum antibody concentration by using ELISA

Ninety six-well microtitre plate (Delta, Nunc) were first coated with 2.5 mg/ml of antibody anti-mouse (against mouse IgG and IgM) suspension in a pH 9.6 NaHCO3-Na2CO3 coating buffer, and stored overnight at 4°C. After 3 washing steps with a pH 7.4 PBS buffer supplemented with Tween 20 (0.05 % final concentration), a 3 % BSA solution was used for 1 h to block the unspecific adsorption sites. This additional plate was coated with BSA. Thereafter the plates were filled with the two-fold serially diluted serum in PBS-Tween 20 (200 µl per well) and incubated at 37°C for 3 hours. After 3 washing steps, perroxidase-conjugated (1µg/ml) anti-mouse antibodies were then added and the plates incubated for one hour at 37°C. Plates were washed again before application of o-phenylenediamine dihydrochloride and oxygen peroxide (200 µl per well).

- NK, T CD4 and T CD8 cells count

Lymphocytes subtypes were determined by flow cytometry on Facscan (Becton Dickinson) by using two colours of immunofluorescence. A negative control was included. 5000 cells were analyzed per sample. Positive fluorescence of the population was determined by the percent of mononuclear cells. Cells were detected by rat monoclonal conjugated with either fluorescein or phycoerythrin. The following cells types were determined: T helper/inducer lymphocytes (CD4, anti-leu-3, Immunotech, France), T suppressor/cytotoxic lymphocytes (CD8, anti-leu-2, Immunotech, France) and NK cell marker (anti-NK1.1, Interchim, France).

2) Microscopical study

- The severity of gastric erosions in stressed mice

Twenty four hours after stress, the abdominal cavity was opened, and stomachs were immediately removed, opened along the greater curvature and washed with solution of tyrode (NaCl: 8 g /KCl : 0.2 g /MgCl2: 0.1 g / NaHCO3 : 1 g /CaCl2 : 0.2g/ Na2HPO4 : 0.05 g /glucose : 1 g/distilled water : 1l).

The Mucosal side of the stomach was photographed by microscopical apparatus

(Nikon, FDX-35.Labophot). The severity score of mucosal erosions was calculated as the summation of the length of erosions (Yano & Harada, 1973). To study the effects of stress on the gastric fabric and cells, we had recourse to the histological technique with systemic standard coloration (Hematoxyline Eosine).

3) Behavioural study

Two tests used to evaluate, locomotory, exploratory and anxious behavioural in mice.

- Light/ dark box test

The apparatus consisted of a Plexiglas box, divided into two compartments (20 x 20 x 20 cm) by a black Plexiglas partition, communicating with a small door, which the animal could easily pass. One of them was darkened; the other was illuminated (Crawley & Goodwin 1980, Costall *et al*. 1989).

Each subject (previously stressed or not) was individually tested in five minutes sessions. Mice were naive to the apparatus and were placed in the lit box to initiate the test session. The amount of time spent in the lit box was recorded each time during 5 min after the first entry of the mice into the dark box. The number of rears in the light side, and the number transitions from dark to light side were recorded over 5 min.

- Staircase test

The apparatus consisted of Plexiglas enclosure (47 x 10 x 25 cm) with five identical steps. The only light source in the room was a 60 watt desk lamp above the staircase (Simiand *et al*., 1984).

The animals were individually placed on the floor of the box; the number of steps climbed and rears performed over a 5 min period were recorded. A step was considered as being climbed when the mouse had placed all four paws on the next upward steps.

Statistical analysis

Data are expressed as means ± S.E.M, statistical differences were calculated by One Way ANOVA and t-test (comparison per pair). Differences between means were considered significant if $p < 0.05$.

Results

Immunological studies

- Leukocyte analysis

Table 1 showed that experimental stress decreased significantly lymphocyte, neutrophiles and basophiles numbers, only at 1 day after immunization. However, the experimental stress showed different effects on monocytes and eosinophiles numbers depending on intervals between immunization and stress, i.e. monocytes number decreased significantly at 1 day, whereas it increased at 3 days after immunization. In contrast, the eosinophiles number increased strongly at 1 day and deceased for all the other intervals.

- Serum antibody concentration

As indicated in table 2, the stressed mice had significantly more anti-SRBC antibodies than unstressed mice (400 %, $P< 0.001$) at 1 day after immunization. However, a decrease of antibodies concentration was observed 5 days after immunization (50 %, $P< 0.001$) when compared to unstressed mice.

- NK, T CD4 and T CD8 cells counts

As showed in figure 1, T CD4 number was decreased significantly into circulation (40 %; $P< 0.001$) at 1 day after immunization in stressed group when compared to unstressed group.

However, we haven't observed any significant variation on T CD8 number at

different intervals of immunization in stressed group with comparison to unstressed one (Figure 2).

In contrast, the number of circulating NK cells was increased at different intervals, principally at 1 day after immunization (370 %, P< 0.001) in stressed group when compared to unstressed one (Figure 3).

Table 1. Effect of experimental stress on the response of leukocytes subpopulations at 1, 3 and 5 days of intervals of immunization in mice. N= 54 mice. IUS: Immunized Unstressed; IS : Immunized Stressed.

Number of cells $10^3/\mu l$	**Lymphocytes** (Mean ± SEM)	**Neutrophiles** (Mean ± SEM)	**Monocytes** (Mean ± SEM)	**Basophiles** (Mean ± SEM)	**Eosinophiles** (Mean ± SEM)
IUS					
1 day	3.820 ± 0.290	1.850 ± 0.401	2.340 ±0.231	0.283 ±0.062	0.040 ± 0.022
3 day	2.812 ± 0.302	0.510 ± 0.061	1.140 ±0.572	0.072 ±0.017	0.011 ± 0.006
5 day	2.695 ± 0.762	1.310 ± 0.281	1.512 ±0.161	0.060 ±0.081	0.051 ± 0.092
One Way ANOVA					
effect of the intervals of the immunization	F(3, 28)= 20.781; P=0.0001.	F(3, 31)= 7.017; p= 0.001.	F(3, 28)= 3.520; p= 0.027.	F(3, 28)= 8.930; p= 0.0003.	F(3, 28)= 3.404; p= 0.031.
IS					
1 day	1.730 ±0.210; p=0.007.	0.901 ±0.281; p= 0.019.	1.950±0.121; p = 0.002.	0.023±0.021; p = 0.001.	1.010±0.210; p = 0.001.
3 day	3.121 ±0.280; p= 0.120.	0.490 ±0.071; p = 0.730.	1.900 ±0.170; p = 0.020.	0.045 ±0.080; p = 0.570.	0.006 ±0.002; p = 0.901.
5 day	2.821 ±0.790; p= 0.610.	1.021 ± 0.101 p = 0.950	1.430 ±0.201; p = 0.790.	0.052 ±0.013; p = 0.801.	0.004 ±0.002; p = 0.600.
Two Way ANOVA					
-Stress effect	F(1,45)=7.389; p =0.009.	F(1,43)=4.125; p = 0.040.	F(1,45)=0.544; p = 0.460.	F(1,38)=9.049; p = 0.0046.	F(1,37)=28.830; p = 0.0001.
- Interval between stress/ immunization	F(2,45)=8.783; p =0.0006.	F(2,43)=12.543; p = 0.0001.	F(2,45)=2.709; p = 0.077.	F(2,38)=11.277; p = 0.0001.	F(2,37)=31.320; p = 0.0001.
-Interaction	F(2,45)=1.056; p = 0.356.	F(2,43)= 2.873; p = 0.067.	F(2,45)=8.170; p = 0.0009.	F(2, 38)=6.870; p = 0.0028.	F(2,37)=34.712; p = 0.0001.

Table 2. Effect of experimental stress on humoral response (i.e. concentration of total antibodies) at 1, 3 and 5 days of intervals of immunization in mice. N= 36. IUS: Immunized Unstressed; IS : Immunized Stressed.

	Number of animals	**Concentration of total antibodies (μg/ml)** (Mean ± SEM)
IUS 1 day	6	9.613 ± 0.872
3 days	6	48.004 ± 2.696
5 days	6	27.562 ± 1.402
One Way ANOVA Effect of interval between immunization and stress		F(3, 36)= 78.734; P= 0.0001.
IS 1 day	6	47.066 ± 3.599; P= 0.0001
3 days	6	40.756 ± 3.125; P= 0.053.
5 days	6	13.059 ± 0.716; P= 0.0001.
Two Way ANOVA		
- Stress effect.		F (1, 57)= 7.393; P= 0.008.
- Immunization effect.		F (2, 57)= 55.046; P= 0.0001.
- Interaction between two factors		F(2, 57)= 66.150; P= 0.0001.

Microscopically studies

The number of erosions per stomach increased after the application of experimental stress. Table 3 shows the distribution of number of erosions per stomach in the stressed group when compared to unstressed one. These results indicate that gastric erosions were developed after experimental stress application at all intervals of immunization. (Photo N°1 and Photo N°2).

We are also observed in Photo N°3 and Photo N°4, a strong gastric inflammation in stressed mice. This inflammation could be explained by an infiltration of polynuclear neutrophiles inside the gastric mucous membrane.

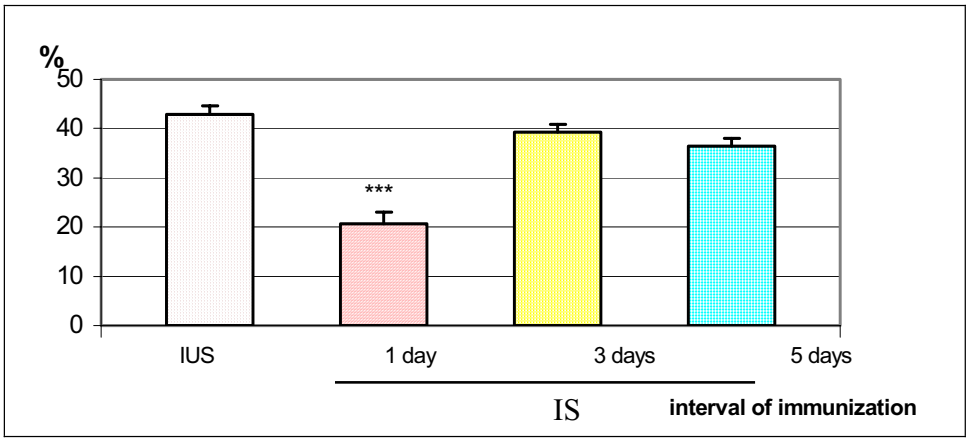

Figure 1. Effect of experimental stress on T helper CD4 number at 1, 3 and 5 days of intervals of immunization in mice. N=40. IUS : Immunized Unstressed, IS: Immunized Stressed. ***: P<0.001.

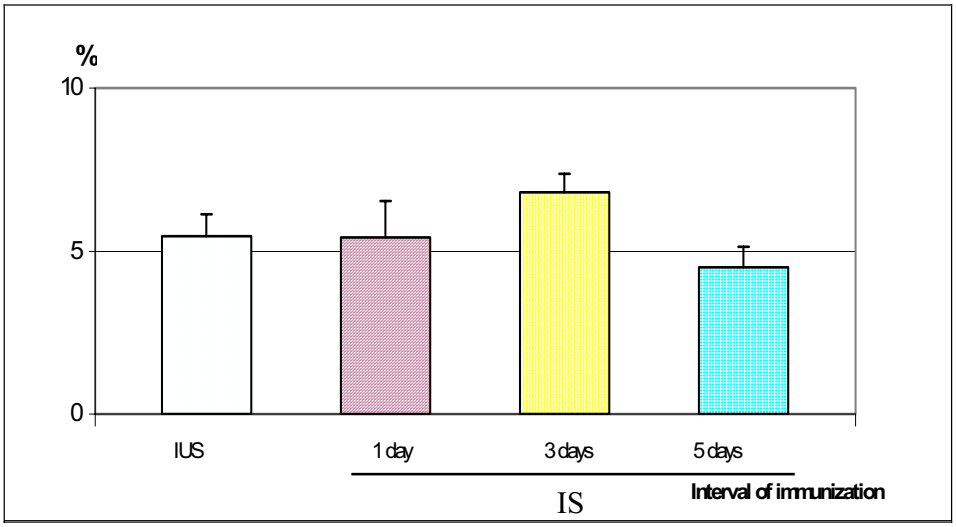

Figure 2. Effect of experimental stress on T cytotoxic/suppressor CD8 number at 1, 3 and 5 days of intervals of immunization in mice. N=40. IUS : Immunized Unstressed, IS: Immunized Stressed.

Behavioural studies

Tables 4 and 5 showed that experimental stress could reduce exploratory locomotor and postural activity in two behavioural tests. The interval separating the immunization and stress did not affect the behavioural parameters (unpublished data).

In fact, in the staircase test, the number of rears and steps climbed decreased (55 % and 40 %; P< 0.01 and P< 0.001 respectively).

In the light/dark box test, time spent in the light compartment increased (55 %, P< 0.001) but the number of rears and transitions from dark to light side decreased significantly (28 %, and 40 %; P< 0.01 and P< 0.001 respectively).

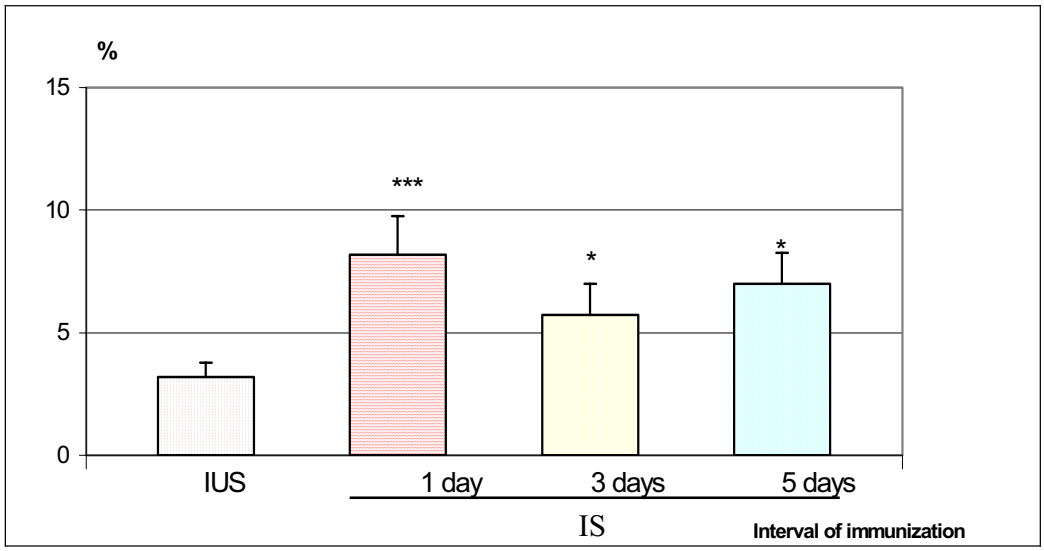

Figure 3. Effect of experimental stress on natural/killer number at 1, 3 and 5 days of intervals of immunization in mice. N=40. IUS : Immunized Unstressed, IS: Immunized Stressed. ***: P< 0.001, *: p< 0.05.

Table 3. Effect of experimental stress on gastric mucous at 1, 3 and 5 days of intervals of immunization in mice. N= 36. . IUS: Immunized Unstressed; IS : Immunized Stressed.

		Number of animals	Mean severity score ± SEM
IUS 1 day		6	3.502 ± 0.320; P= 0.541.
	3 days	6	3.120 ± 0.300; P= 0.446.
	5 days	6	3.741 ± 0.101; P= 0.823.
One Way ANOVA			
Effect of interval between immunization and stress			F(3, 33)= 2.449; P= 0.081.
IS 1 day		6	7.601 ± 0.250; P= 0.0001.
	3 days	6	7.102 ± 0.721; P= 0.0001.
	5 days	6	6.403 ± 0.812; P= 0.005.
Two Way ANOVA			
- Stress effect.			F(1, 58)= 1.460; P= 0.0001.
- Immunization effect.			F(2, 58)= 0.874; P= 0.422.
- Interaction between two factors			F(2, 58)= 13.024; P= 0.0001.

Discussion and conclusion

Few studies consider the importance of time intervals separating immunization, application of experimental stress and time of sacrifice. Our results obtained with the experimental stress model (footshock stress) in mice, shows that effects on the immune system are largely dependent on the duration and intervals of the stressor applied following immunization.

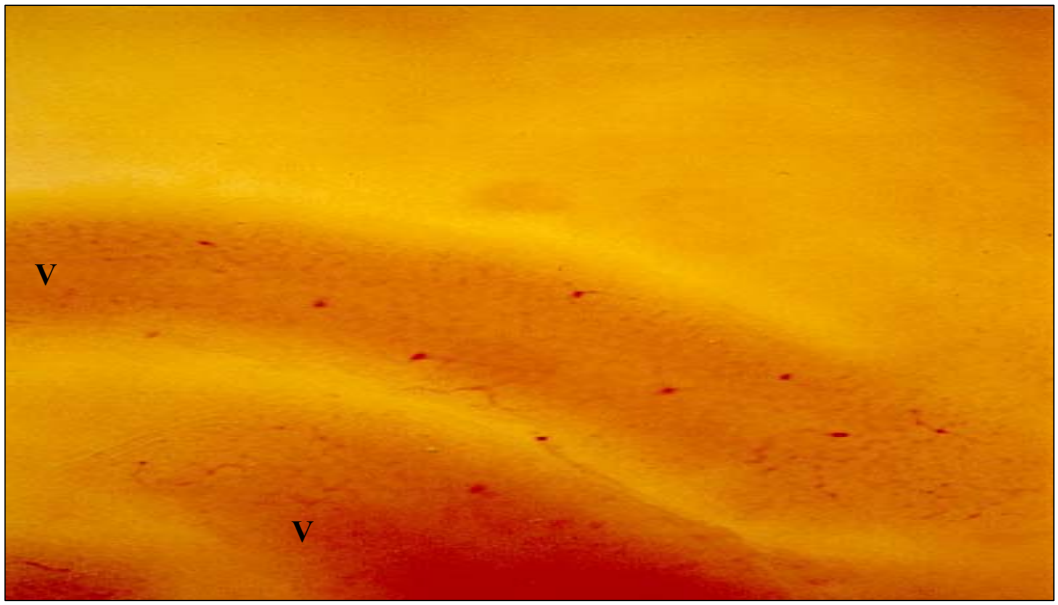

Photo 1. Microphotograph of the stomach villosity displays a few gastric erosions 'red points', 1 day after immunization (x60). V: villosity.

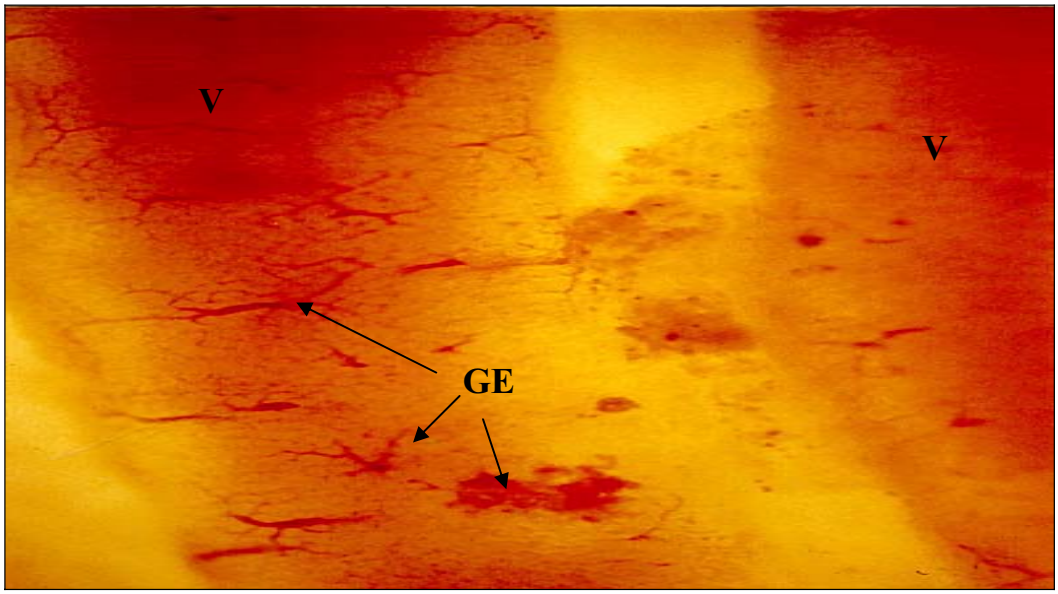

Photo 2. Microphotograh of the stomach of stressed mouse (x32). Severe gastric erosions are observed after stress exposure, 1 day after immunization. V: villosity; GE: gastric erosions.

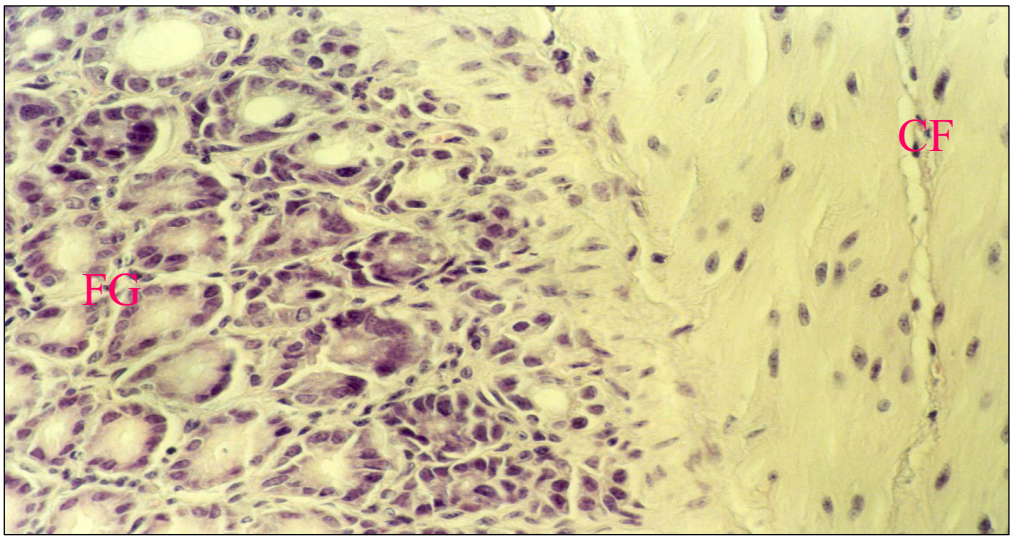

Photo 3. Histological cuts of a stomach of unstressed mouse, 1 day after immunization. CF: conjunctive fabric. FG: fundic glands.

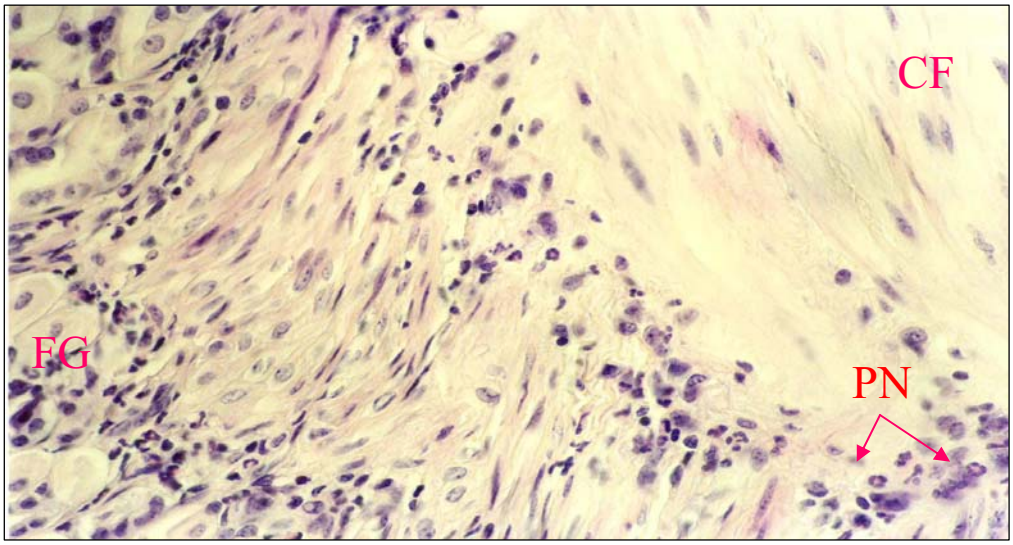

Photo 4. Histological cuts of a stomach of stressed mouse, 1 day after immunization. CF: conjunctive fabric. FG: fundic glands. PN: polynuclear neutrophiles.

In our data, the leukocyte number in the peripheral blood was significantly decreased, 1 day after immunization in stressed mice except for eosinophiles which increased significantly at this period. It's had demonstrated that restraint stress caused a significant decrease in the leukocytes number which due to a decrease in the number of the peripheral lymphocytes, whereas the neutrophils were unchanged (Millan *et al*., 1996).

In accordance with our study, physical pain (Fujiwara & Orita, 1987), electric footshock (Croiset *et al*., 1987), psychological stress (Edwards *et al*., 2006) and restraint stress (Berkenbosch *et al*., 1991) induce an increase on humoral immune response. It was observed similar time-response patterns for eosinophiles and

serum concentration of total antibodies, both displaying a concomitant peak 1 day after immunization (responses increased by 2 and 5 fold respectively) followed by a decrease

Table 4. Effect of experimental stress on transitions, rears and time spent in the lit compartment of the light/dark box test in mice. N=20.

	Number of animals	Rears/lit	Time/lit (en sec)	Transitions
US				
Mean ± SEM	10	27.126 ± 1.400	91.900 ± 9.395	10.800 ± 0.860
S				
Mean ± SEM	10	18.000 ± 1.020	177.700 ±18.372	6.857 ± 0.459
t-test		*t-test*= 7.946 ; P=0.0001	*t*-test= -6.386; P=	*t*-test= 6.199;
P			0.0001.	P=0.0001

Table 5. Effect of experimental stress on rears and steps climbed in staircase test in mice. N= 20.

	Number of animals	Rears	Steps climbed
US			
Mean ± SEM	10	22.625 ± 1.782	27.750 ± 2.177
S			
Mean ± SEM	10	16.571± 2.716	13 ± 3.078
t-test		t-test=2.954;	t-test=4.03;
P		P=0.012	P=0.001

which becomes highly significant (P< 0.001) 5 days after immunization. Such observations have also been reported for hypersensitivity phenomena (Janneway & Travers, 1997).

Concerning the sub-populations lymphocytes, the work of Okimura & Nigo, (1986) suggests that T and B cells have the same sensitivity to the stress in the decrease of cells number. Our results noted that T CD4 number decreased but number of NK cells increased into circulation. No change in T CD8 number was seen after stressor exposure.

Many studies have noted effects of stress on immune functions; typical changes include lymphocytosis as well as more functional changes such as an increase in natural killer cell number and cytotoxicity (Naliboff *et al*. 1991, Cacioppo 1994). Other studies noted that T CD8 and NK numbers in the peripheral circulation typically increases (Herbert *et al*. 1994, Sgoutas-Emch *et al*. 1994). In contrast, T helper/inducer CD4 decrease into circulation after acute psychological stress (Marsland *et al*., 1997).

The study of Maddock & Pariante (2001) reported that chronic stress appears to result in suppression of the immune response, whereas immune activation and suppression have been associated with acute stress. Other works of [4, 29] reported that both helper T cells and suppressor T cell activities were inhibited but B cells activities were enhanced after restraint stress.

The present results also demonstrated that stress induced a significant increase in absolute number of natural killer cells. These changes are often attributed to processes such as lymphocyte migration from lymphoïd organs to circulating blood or demargination of endothelium adherent lymphocytes (Van Tits *et al*. 1990, Benshop *et al*. 1993); the β adrenergic

receptors have been identified on NK, CD4 and CD8 cells with the highest receptor density on NK cells (Khan *et al*., 1986). It is possible, however that lymphocyte populations could not increase under stress but instead rise only in their relative blood concentration due to a concomitant reduction in plasma volume. Increases in T CD8 and NK cells following stress were partly attributable to plasma volume diminution and CD4 number decreases after stress exposure when hemoconcentration is taken into account (Pattersson *et al*. 1995, Marsland *et al*. 1997). Others authors noted that in animals, acute stress usually drives the immune response towards a Th2, grossly "immunosuppressive profile". In humans, acute stress associates an endocrine response (characterized by glucocorticoid secretion and hyperprolectinemia) with an immunosuppression (Vuitton *et al*., 1999).

The stress model used in the present study caused a greatest severity of gastric erosions also 1 day after immunization, these results were in accordance with the study of Hernandez (1986) and Yano & Harada (1973).

We observed that our model of stress decreases significantly the behavioural parameters in the two tests (Light/dark box test and staircase test). Our data confirmed the results of other authors who demonstrated that restriction of environmental space (Mitsushima *et al*., 1998), novelty (Aloisi *et al*., 1997) attenuates locomotor activity.

In conclusion, the present study suggest that exposure to stress (repetitive and unavoidable) can alter simultaneously the integrated outcome of behaviour, gastric and immune response to an antigen in mice. These responses are characterized by an increase in NK cells and in total antibodies of serum concentration, by a decrease in the leukocyte number in peripheral blood (except for eosinophiles, which increased significantly), and a decrease of helper/inducer TCD4 cells. However suppressor/cytotoxic TCD8 are not affected by stress in our experimental conditions. The experimental stress showed impairment in exploratory and locomotory behaviours of mice subjected to light/dark and staircase tests.

The number and severity and severity of gastric erosions increased after stress exposure, due partly to the infiltration of polynuclear neutrophiles into gastric mucous. This finding confirms a decrease of polynuclear neutrophiles into peripheral circulation.

Acknowledgements

We thank Dr Nicolas FROMENT for consultations about Flow cytometry technique and for his generous support.

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