Physiol. Res. 51: 291-298, 2002

Comparative Study of Several Lymphocyte Functions in Two Strains of Mice with Different Models of Endotoxic Shock

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Received July 30, 2001
Accepted September 26, 2001

Summary

Previously, the changes in phagocyte functions such as adherence, chemotaxis or TNFα production were found to be associated with oxidative stress in endotoxin-induced septic shock. However, in this type of oxidative stress the lymphocyte involvement has rarely been studied. In the present report, we analyzed the above functions in peritoneal lymphocytes from male and female BALB/c mice with a lethal endotoxic shock caused by intraperitoneal injection of *E. coli* lipopolysaccharide (LPS) (100 mg/kg), male and female Swiss mice with lethal endotoxic shock caused by intraperitoneal injection of LPS (150 and 250 mg/kg, respectively) or non-lethal endotoxic shock (100 mg/kg). In peritoneal lymphocytes obtained at 0, 2, 4, 12 or 24 h after LPS injection, the first two functions of these cells in the immune response, i.e. adherence to tissues and directed migration (chemotaxis), were studied. At 0, 0.5, 1, 1.5, 2, 4, 12 and 24 h after LPS injection, TNFα released by lymphocytes was also analyzed. The results show that endotoxic shock increases the adherence and TNFα release, and decreases the chemotaxis of peritoneal lymphocytes. These changes were more significant in mice with lethal than with non-lethal endotoxic shock, a fact that confirms the important role of lymphocytes during endotoxic shock.

Key words
Lymphocyte functions • Endotoxic shock • Mice

Introduction

Lipopolysaccharides (LPS), which are a component of the cell envelope of gram-negative bacteria, are the most frequent causative agent of septic shock (Glauser *et al*. 1991, Danner *et al*. 1991). Exposure of immune cells, such as phagocytes, to LPS results in the intense production of reactive oxygen species (ROS) (Víctor *et al*. 1998, Spolarics 1998, Schafer *et al.* 1999), that are essential for infection containment. However, excessive activation of these cells may induce oxidative damage and resulting high mortality and morbidity, as has been observed in sepsis (Morrison and Ryan 1987, Nicholson *et al.* 1999). This is linked to the fact that, activation of immune cells by endotoxin results in the production of a number of proinflammatory cytokines, such as the tumor necrosis factor alpha (TNFα), the major proinflammatory cytokine involved in endotoxic shock (Beutler *et al*. 1985, Laskin and Pendino 1995). In fact, the administration of antibody antiTNFα or knockout of TNFα receptor greatly diminished or abrogated mortality in endotoxic models (Tracey *et al.* 1987). We have previously observed that peritoneal macrophages from BALB/c mice with lethal endotoxic

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shock show a high production of superoxide anion and TNFα as well as changes in functions related with oxidative stress such as adherence, chemotaxis or ingestion (Víctor *et al*. 1998, 1999). Moreover, these changes were more significant in macrophages from mice with lethal endotoxic shock than in those from mice with non-lethal endotoxic shock (Víctor *et al.* 2000).

Although the role of phagocytic cells in oxidative stress induced by endotoxin seems to be evident, there are only a few studies about the implication of lymphocytes in that process (Traber *et al*. 1983, De la Fuente and Víctor 2001). In view of the above, the aim of the present study was to investigate the changes in two initial activities of lymphocytes in the immune response i.e. adherence to tissue and directed migration (chemotaxis), which are essential for immune surveillance and for promoting cell-to-cell interactions involved in the immune response (Carlson *et al.* 1997), as well as TNFα production. It was studied in male and female mice of two strains, BALB/c and Swiss, with lethal and non-lethal endotoxic shock.

Methods

Materials

The lipopolysaccharide (*Escherichia coli* LPS 055:B5) and f-met-leu-phe were purchased from Sigma (St. Louis, MO, USA), filters of nitrocellulose from Millipore (Bedford, MA, USA), trypan blue from Merck (Darmstadt, Germany), tumor necrosis factor α (TNFα) immunoassay was obtained from Endogen (Woburn, MA, USA), RPMI 1640 medium from Gibco (Paisley, Scotland), and 96 well plates were purchased from Costar (Badhoevedorp, The Netherlands).

Animals

Adult BALB/c and Swiss (OF-1) mice (Harlan Interfauna Ibérica, Barcelona, Spain), aged 24±2 weeks old were used, since previous work from our laboratory has shown that at that age the mice show no signs of immune function decline (Víctor et al. 1998). The same age was used in our previous study on BALB/c mice.

The mice were maintained at a constant temperature (22±2 ^{o}C) in a reverse 12:12 dark light cycle, with free access to food (A04 diet from Panlab L.S. Barcelona, Spain) and water. The animals used did not show any signs of malignancy or other pathological processes. Mice were treated according to the guidelines of the European Community Council Directive 86/6091 EEC.

Experimental protocol

Six groups of 8 animals each were used. Lethal endotoxic shock was induced by intraperitoneal injection (i.p.) of LPS at a concentration of 100 mg/kg in male and female BALB/c mice, and 150 mg/kg in male Swiss, and 250 mg/kg in female Swiss mice. Non-lethal endotoxic shock was induced at a concentration of 100 mg/kg in male and female Swiss mice. Each animal was injected between 9:00 a.m. and 10:00 a.m. We analyzed the functions of the peritoneal lymphocytes from 6 animals (one from each group) every day for three weeks.

Although we have previously observed that the oestrous cycle phase of the female mice has no effect on this experimental assay, all females used in the present study were at the beginning of diestrus.

Mortality experiment

In order to determine the lethal and non-lethal effect of the different concentrations of LPS studied, a group of 12 mice per group was used for observation of the mortality after endotoxin administration.

Collection of peritoneal exudate cells

At 0, 2, 4, 12 and 24 h after injection, peritoneal suspensions were obtained by a procedure previously described (De la Fuente 1985). Briefly, 3 ml of Hank's solution, adjusted to pH 7.4, were injected intraperitoneally, then the abdomen was massaged and the peritoneal exudate cells, consisting of lymphocytes and macrophages, were collected allowing recovery of 90-95 % of the injected volume. Lymphocytes, identified by morphology and cytometric assay, were counted and adjusted in Hank's solution to $1x10^{6}$ lymphocytes/ml. Cellular viability was routinely measured before and after each experiment by the trypan-blue exclusion test. The viability was higher than 95 % in all cases. All incubations were performed at 37 ^{o}C in a humidified atmosphere of 5 % CO_2.

Assay of adherence capacity

The quantification of substrate adherence capacity was carried out by a method previously described (De la Fuente *et al.* 1994). Aliquots of 200 µl of peritoneal suspension were placed in Eppendorf tubes and, after 10 min of incubation, 10 µl were removed from

each sample after gently shaking to resuspend the sedimented cells. The number of non-adhered lymphocytes was determined by counting in Neubauer chambers under an optical microscope (x40 magnification lens). The adherence index, AI, was calculated according to the following equation:

AI = 100 – [(lymphocytes/ml supernatant) / (lymphocytes/ml original sample)] x 100

Assay of chemotaxis

The mobility directed to a chemo-attractant gradient (chemotaxis) was evaluated according to the method of De la Fuente *et al.* (1994). It consists basically of the use of chambers with two compartments separated by a filter, with a pore diameter of 3 µm. Aliquots of 300 µl of peritoneal suspension were deposited in the upper compartment and aliquots of 400 µl of f-met-leu-phe (fMLP; 10^{-8} M), a well-known chemo-attractant used in previous studies, were put into the lower compartment. The chambers were incubated for 3 h, and then the filters were fixed and stained. The chemotaxis index was determined by counting the total number of lymphocytes on one-third of the lower face of the filter, corresponding to four scans of 8 mm, using an optical microscope (x100 magnification).

TNFα production

The mouse tumor necrosis factor alpha (TNFα) production was determined on culture supernatants using a commercial mouse TNFα immunoassay. The sensitivity limit of the assay was 10 pg/ml. To obtain culture supernatants, aliquots of 200 µl of lymphocytes purified by adherence of the peritoneal suspension (adjusted to $1x10^6$ lymphocytes/ml of RPMI medium) were dispensed in plates of 96 wells. After 24 h of incubation, the plates were centrifuged at 1200 rpm for 10 min and the supernatants were obtained and maintained in a freezer at -80 ^oC until assay of TNFα.

Expression of the results and statistical analysis

The data are expressed as the mean ± standard deviation (S.D.) of the values from the number of experiments shown in the tables and figures. The data were analysed by one-way repeated measures analysis of variance (ANOVA) since data did not show Gaussian distribution in the different groups of mice at 0, 2, 4, 12 and 24 h after LPS injection. Multiple ANOVA was used to compare the values obtained between strains and doses. The Student Newman Keuls test with a level of significance set at $p<0.05$ was used for *post hoc* comparisons.

Table 1. Mortality of animals after LPS injection

Time (h) after LPS injection	**Swiss mice**				**BALB/c mice**	
	Male		**Female**		**Male**	**Female**
	100 mg/kg	**150 mg/kg**	**100 mg/kg**	**250 mg/kg**	**100 mg/kg**	**100 mg/kg**
0-12	0	16	0	0	0	0
13-20	0	32	0	16	16	15
20-24	0	64	0	32	50	65
24-30	0	100	0	100	100	100

Each value represents the accumulated percentage of mortality of animals after injection of LPS. The number of animals used was 12 per group.

Results

Mortality

Table 1 shows the accumulated percentage of mortality of Swiss and BALB/c mice at different times after administration of different doses of LPS. The results show that Swiss mice injected with 150 mg/kg (male) or 250 mg/kg (female), and male and female BALB/c mice injected with 100 mg/kg, exhibited a mortality rate of 100 % at 30 h (lethal shock groups). In male and female Swiss mice, injected with 100 mg/kg of LPS, no mortality was observed at this time, with 100 % survival of the animals (non-lethal shock groups).

Adherence capacity

Figure 1 shows the adherence indexes (A.I.) of peritoneal lymphocytes at 10 min of incubation, from animals after 0, 2, 4, 12 and 24 h of LPS injections. At zero time, the AI values in the two strains of mice were higher in Swiss than in BALB/c mice. In the lethal shock

groups, the AIs were increased significantly. When comparing lethal groups, the results obtained in the male groups and in the female groups, the effect of LPS in AIs was in general significantly increased in the males, mainly at 24 h.

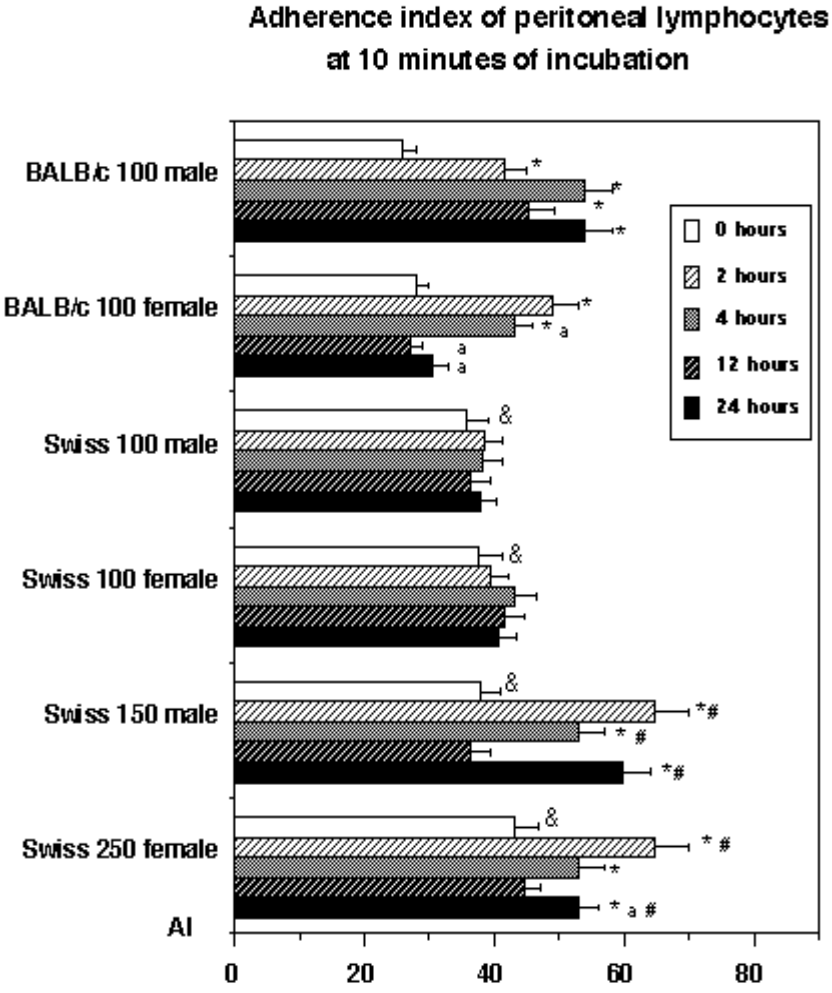

Fig. 1. Adherence imdexes (AI) at 10 min of incubation of peritoneal lymphocytes. Lethal endotoxic shock was induced by intraperitoneal injection of LPS at a concentration of 100 mg/kg in male and female BALB/c mice, and of 150 mg/kg and 250 mg/kg in male and female Swiss mice, respectively. Non-lethal endotoxic shock was induced at a concentration of 100 mg/kg in male and female Swiss mice. The cells were obtained at 0, 2, 4, 12 and 24 h after LPS injection. Each column represents the mean ± SD of 8 values corresponding to 8 animals, each value being the mean of duplicate assays. *$p<0.05$ respect to the values at 0 h (control). $^{a}p<0.05$ with respect to the corresponding female group. #$p<0.05$ between lethal and non-lethal groups in Swiss mice. &$p<0.05$ between Swiss and BALB/c mice at 0 h after injection.

Chemotaxis capacity

Figure 2 shows the chemotaxis index (CI) of peritoneal lymphocytes from animals after 0, 2, 4, 12 and 24 h of LPS injections. Comparing the CI obtained at 0 h between BALB/c and Swiss mice, the values were higher in BALB/c than in Swiss mice. In all groups, the CIs were decreased significantly at all times except in male and female Swiss mice of the non-lethal group at 12 and 24 h. These decreases were higher at all times after LPS injection in cells from animals with lethal endotoxic shock. Comparing the results obtained in the male groups with those of the female groups, these decreases were higher in the male BALB/c mice at 24 h.

TNFα amount release

The TNFα release by peritoneal lymphocytes from the BALB/c and Swiss groups at 0.5, 1, 1.5, 2, 4, 12 and 24 h after LPS injection is shown in Fig. 3. At zero time, there is no difference between the different groups. The TNFα amounts increased in Swiss mice at the beginning of endotoxic shock and the highest levels were shown at 1.5 h, while the increase in BALB/c mice occurred mainly at 2 h. After that, TNFα levels decreased, but they remained higher in the lethal groups than in non-lethal groups. With respect to the differences between males and females, in general, the levels of TNFα were higher in males than in females BALB/c mice, while the opposite effect was observed in Swiss mice.

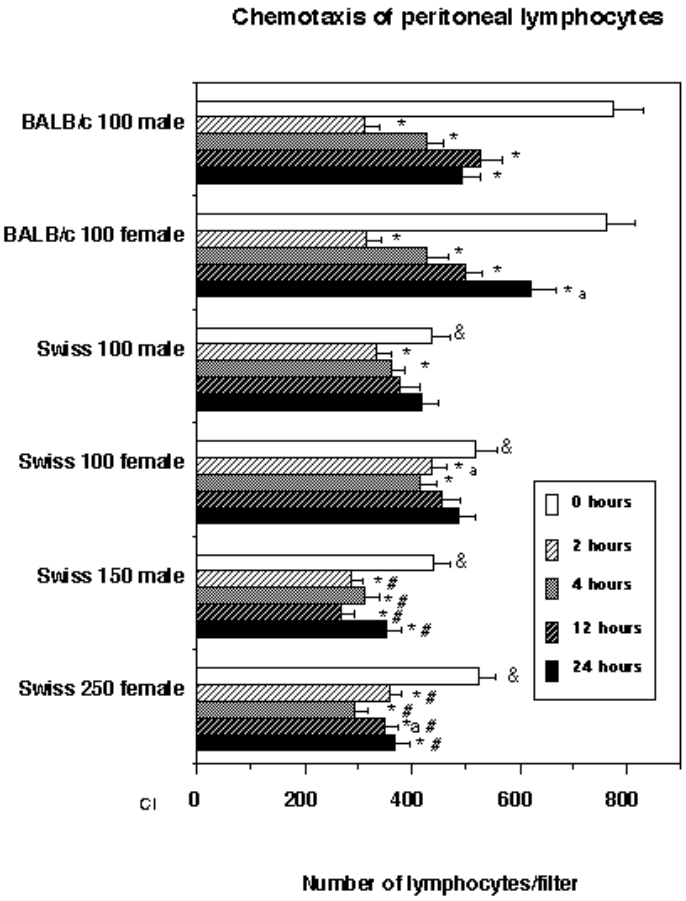

Fig. 2. Chemotaxis indices (CI) of peritoneal lymphocytes. Lethal endotoxic shock was induced by intraperitoneal injection of LPS at a concentration of 100 mg/kg in male and female BALB/c mice, and of 150 mg/kg and 250 mg/kg in male and female Swiss mice, respectively. Non-lethal endotoxic shock was induced at a concentration of 100 mg/kg in male and female Swiss mice. The cells, were obtained at 0, 2, 4, 12 and 24 h after LPS injection. Each column represents the mean ± SD of 8 values corresponding to 8 animals, each value being the mean of duplicate assays. *p<0.05 respect to the values at 0 h (control). [a]p<0.05 with respect to the corresponding female group. #p<0.05 between lethal and non-lethal groups in Swiss mice. &p<0.05 between Swiss and BALB/c mice at 0 h after injection.

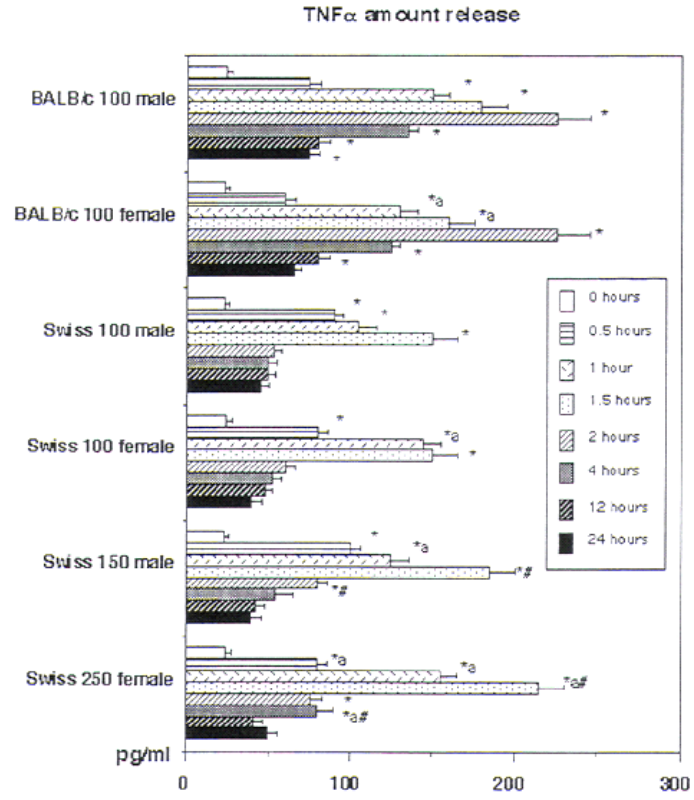

Fig. 3. TNFα amount release (pg/ml) of peritoneal lymphocytes. Lethal endotoxic shock was induced by intraperitoneal injection of LPS at a concentration of 100 mg/kg in male and female BALB/c mice, and of 150 mg/kg and 250 mg/kg in male and female Swiss mice, respectively. Non-lethal endotoxic shock was induced at a concentration of 100 mg/kg in male and female Swiss mice. The cells were obtained at 0, 0.5, 1, 1.5, 2, 4, 12 and 24 h after LPS injection. Each column represents the mean ± SD of 8 values corresponding to 8 animals, each value being the mean of duplicate assays. *p<0.05 with respect to the values at 0 h (control). [a]p<0.05 with respect to the corresponding female group. #p<0.05 between lethal and non-lethal groups in Swiss mice. &p<0.05 between Swiss and BALB/c mice at 0 h after injection.

Discussion

This study shows the unfavourable changes occurring in several functions of peritoneal lymphocytes, i.e. adherence to tissues, migration directed to the antigen focus (chemotaxis) and TNFα release, in cells from male and female BALB/c and Swiss mice with lethal and non-lethal endotoxic shock.

Adherence and migration are two functions shared by macrophages and lymphocytes that represent the earliest events in the immune response (Unanue and Allen 1987). We have observed in our previous studies changes in these functions, i.e. an increase in adherence and a decrease in chemotaxis of peritoneal macrophages from BALB/c mice with lethal endotoxic shock caused by intraperitoneal injection of E. coli LPS (100 mg/kg) (Víctor *et al*. 1998). In these animals, a high production of superoxide anion by macrophages (Víctor *et al*. 1998) and TNFα (Victor *et al*. 1999) was also shown, a fact that supports the oxidative stress situation in these animals**.** The results of the present study show that the adherence capacity and TNFα release in peritoneal lymphocytes are enhanced whereas the chemotaxis is decreased after the injection of LPS in male and female BALB/c and Swiss mice. Moreover, all these changes were more evident in lethal than in non-lethal endotoxic shock groups.

The stimulation of adherence, an early function in the immune response of lymphocytes after endotoxin injection, could be due to the increased adhesion molecular expression that occurs under oxidative stress conditions (Hmama *et al.* 1999). Is is known that the increase of TNFα and ROS production by LPS stimulation is involved in a higher adhesion molecule expression (Baveye *et al.* 2000). This stimulation of the adherence capacity has been shown by phagocytes and lymphocytes in other oxidative stress situation such as aging (Ortega *et al*. 2000).

The next step in the immune response, namely chemotaxis, was significantly inhibited after LPS injection. This effect is probably a consequence of the production of the migration inhibitory factor (MIF) in response to LPS (Calandra and Bucala 1997). This decrease of the chemotaxis capacity has been observed in oxidative stress conditions such as strenous exercise (Ferrández and De la Fuente 1999), aging (Ortega *et al.* 2000) or with a deficit of antioxidants in the diet (De la Fuente *et al*. 2000).

All these changes, the increase in adherence and the decrease in chemotaxis, were more evident in mice with lethal endotoxic shock, and quite similar to those obtained in previous work on macrophages from BALB/c mice with lethal shock in which an oxidative stress situation had been detected (Víctor *et al.* 1998).

The TNFα plays an important role in the pathogenesis of endotoxic shock (Vassali 1992), and the present research shows that its levels are higher in lethal than in non-lethal shock groups. Lymphocytes from the Swiss in comparison with those from the BALB/c mice show different kinetics of TNFα release as we have also observed in macrophages (unpublished data).

Lymphocytes play an essential role in the immune response linked to inflammatory and infectious processes (Traber *et al.* 1983), and they generate ROS and cytokines (Muller *et al*. 1997). Since lymphocytes bind endotoxins (Dziarski 1991), which act as super-antigens causing massive lymphocyte activation (Chance 1996), LPS can either directly or through monokine production by phagocytes act on lymphocytes, which release ROS (Muller *et al.* 1997) and lymphokines as IFNγ (Ma *et al.* 1997) or TNFα (as the present work has shown), thus leading to increased oxidative stress.

In summary, the data suggest, in agreement with Traber *et al.* (1983), that lymphocytes play an important role in host response during endotoxic shock, thus contributing to the oxidative stress implicated in the pathogenesis of this process. In the present work, peritoneal lymphocytes show an increase of adherence capacity, TNFα release and a decrease of chemotaxis after LPS injection, typical behavior of immune cells under oxidative conditions. Thus, phagocytes would not be the only cells implicated in the oxidative stress caused by endotoxin, since lymphocytes are also involved. Therefore the attenuation of the toxic effects of lymphocyte products can reduce the extent of LPS-induced injury.

Acknowledgements

This work was supported by the MCYT (BFI 2001-1218) and by the Comunidad de Madrid grants (085/0061/2002.2).

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Reprint requests

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